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ABSTRACT

My thesis examines the comparative aspects of female reproductive biology in mammals and the origins of pregnancy-specific signaling. I focus on the evolution of three critical reproductive phases: the luteal phase of the ovarian cycle, implantation, and the final establishment of a fetal-maternal interface. These systems show a transition from maternal-only signaling to one involving the placenta, with the latter changing the endocrine control of pregnancy and entailing the establishment and maintenance of a newly formed tissue. I identify the signaling milestones vital to the evolution of eutherian pregnancy, from signals for maternal recognition of pregnancy to the cell-cell communication between maternal and trophoblast cells in a phylogenetic context. In Part I, I analyze the evolutionary connection between reproductive and non-reproductive cycles in vertebrates, focusing on the evolution of longer gestation lengths in mammals. Secondly, I explore the history and evolutionary theories of the female orgasm and its relevance to the concept of homology in comparative biology. In Part II, I present two comparative single-cell gene expression studies. First, I present a study of the cell type inventory and communication dynamics of the fully-formed utero-placenta interface of two rodents with different pregnancy types, mouse and guinea pig, in contrast with the opossum, a primarily non-invasive species, the tenrec, a basally branching eutherian. Lastly, I present a comparison of single-cell gene expression of the mouse and guinea pig with the opossum at the time of initial embryo attachment, uncovering signaling underlying the implantation reaction. Together, my thesis explores the continuities and discontinuities in the evolution of signaling across major innovations in mammalian female reproductive biology.

ZUSAMMENFASSUNG

In meiner Dissertation vergleiche ich die Fortpflanzungsbiologie weiblicher Säugetiere und die Ursprünge schwangerschaftsspezifischer Signalübertragung. Ich konzentriere mich dabei auf die evolutionäre Entwicklung dreier kritischer Reproduktionsphasen: die Lutealphase des ovariellen Zyklus, die Einnistung der Eizelle, und die Etablierung einer fötal-maternalen Schnittstelle. Diese Systeme zeigen einen Übergang von einer rein mütterlichen Signalgebung zu einer, die die Plazenta mit einbezieht, wobei letztere die endokrine Kontrolle der Schwangerschaft verändert und die Etablierung und Aufrechterhaltung eines neu gebildeten Gewebes mit sich bringt. Ich identifiziere die Meilensteine der Signalübertragung, die für die Evolution der Eutheria-Schwangerschaft entscheidend sind, von Signalen für die mütterliche Erkennung der Schwangerschaft bis hin zur Zell-Zell-Kommunikation zwischen mütterlichen und trophoblastischen Zellen in einem phylogenetischen Kontext. In Teil I analysiere ich den evolutionären Zusammenhang zwischen reproduktiven und nicht-reproduktiven Zyklen bei Wirbeltieren und konzentriere mich dabei auf die Entwicklung längerer Trächtigkeitszeiten bei Säugetieren. Zweitens untersuche ich die Geschichte und die evolutionären Theorien des weiblichen Orgasmus und seine Bedeutung für das Konzept der Homologie in der vergleichenden Biologie. In Teil II stelle ich zwei vergleichende Studien zur Genexpression in Einzelzellen vor. Zunächst stelle ich eine Untersuchung des Zelltypeninventars und der Kommunikationsdynamik der voll ausgebildeten Uterus-Plazenta-Schnittstelle zweier Nagetiere mit unterschiedlichen Schwangerschaftstypen vor, nämlich der Maus und des Meerschweinchens, im Gegensatz zum Opossum, einer primär nicht-invasiven Art, und dem Tenrec, einem basalen Eutherier. Schließlich stelle ich einen Vergleich der Einzelzell-Genexpression von Maus und Meerschweinchen mit der des Opossums zum Zeitpunkt der ersten Anheftung des Embryos vor, um die der Einnistungsreaktion zugrunde liegende Signalgebung aufzudecken. Insgesamt erforscht meine Arbeit die Kontinuitäten und Diskontinuitäten in der Evolution der Signalübertragung bei wichtigen Innovationen in der weiblichen Fortpflanzungsbiologie von Säugetieren.

OVERVIEW OF PUBLICATIONS

Paper 1. The shifting role and regulation of the corpus luteum in vertebrate reproduction: a synthetic review.

Authors: **Silvia Basanta** and Mihaela Pavlicev.

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Paper 2. The female orgasm and the homology concept in evolutionary biology.

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Paper 3. Cell type and cell signaling innovations underlying mammalian pregnancy.

Authors: Daniel J. Stadtmauer*, **Silvia Basanta Martínez***, Jamie D. Maziarz, Alison G. Cole, Gülay Dagdas, Gilbecca Rae Smith, Frank van Breukelen, Mihaela Pavličev, Günter P. Wagner.

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Paper 4. Hallmarks of uterine receptivity predate placental mammals.

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PART I

THE THREE SYSTEMS:

EVOLUTION OF PREGNANT -SPECIFIC SIGNALING

Cell-cell signaling permeates every aspect of female reproductive biology. In each cycle, the endocrine system, which involves the production and secretion of hormones, regulates cyclical changes in the reproductive system and the endometrium. Hormones orchestrate changes in paracrine communication between dynamic, resident, and recruited endometrial cells. In placental mammals, functional communication networks between fetal and maternal cells are established during pregnancy, and the placenta becomes an additional exogenous source of endocrine signaling.

Pregnancy in placental mammals is a complex physiological phenomenon involving multiple processes and events, including ovulation, fertilization, placentation, decidualization, embryonic growth, and parturition. Amidst this web of events, three salient systems stand out: the luteal phase of the ovarian cycle, implantation, and the final establishment of a fetal-maternal interface. The luteal phase, a period of high progesterone production by the ovary, is crucial in preparing the uterus for a potential pregnancy. Implantation entails the attachment of the blastocyst to the uterus wall, thus constituting the first direct cell-cell communication between fetal and maternal tissues. Lastly, the fetal-maternal interface is the stable, homeostatic contact of these two tissues. The transition from maternal-only signaling to one involving the placenta marks a pivotal moment in the evolution of pregnancy. This shift changes the endocrine control of pregnancy and establishes a newly formed tissue composed of cells from different origins. Although pregnancy constitutes a character complex, comprising many traits that can vary independently, conceptualizing it in connection to these three semi-independent systems can shed new light on evolutionary and comparative approaches to female reproductive biology.

Thinking of these three systems in terms of signaling transitions underlies the chapters of my thesis. Signaling milestones are vital to the evolution of eutherian pregnancy, from signals for maternal recognition of pregnancy to the specialized, intimate cell-cell communication between maternal and trophoblast cells. On the one hand, a signaling innovation in pregnancy can shift the signaling source from maternal to embryonic or vice versa. These innovations replace or double signals previously maternal by placental sources later in gestation. On the other hand, signaling innovations can be *truly new* and specific to pregnancy. Embryo implantation is a distinctive hallmark of eutherian reproductive biology. Before that, another unique feature of eutherian pregnancy, maternal recognition of pregnancy (MRP), entails signals from the early embryo that rescue the maternal corpus luteum lifespan, turning it into a corpus luteum of pregnancy that will keep producing progesterone beyond the duration of a luteal phase. MRP is thus the first signaling event with the potential to shift maternal physiology and transform it into *pregnant* physiology.

In non-mammalian species, the whole gestation process, from fertilization of the ovum to life birth, happens under the maternal endocrine control and within the length of a luteal phase. Therefore, viviparity and gestation in non-mammalian species occur primarily within the confines of a pre-implantation system. Likewise, most of the gestation process happens during the pre-implantation phase in marsupials, with a short and analogous process to implantation triggering parturition, thus only allowing for a brief fetal-maternal connection **(Figure 1.1)**. A long post-implantation system is only a distinctive and critical factor in the evolution and diversity of eutherian mammals. Within eutherians, the endocrine control of gestation relies on the corpus luteum and its associated hormones until the placenta takes over progesterone production **(Figure 1.2a)**. This last transition, known as the luteal-placental shift, is also crucial to eutherian reproductive biology. Although all eutherian mammals require progesterone throughout gestation, abundant variation exists regarding this endocrinological axis. Eutherian species present luteal-placental shifts in hormonal production at

different stages of gestation. This shift never occurs in other species, particularly in those with short gestation lengths **(Figure 1.2b)**. In some eutherians, gestation still happens within the length of the luteal phase without the need for maternal recognition of pregnancy **(Figure 1.2c)**, which likely constitutes a derived feature.

Stages of gestation before the luteal-placental shift are expected to be more homologous than those in later stages. The mid-to-late maintenance of pregnancy, that is, gestation once the placenta is fully formed and allows the fetus to continue growing within the uterus, typical of precocial species, likely depends on different mechanisms that evolved independently (Chavan et al., 2016). It is also worth mentioning that the immune system profile, particularly the inflammatory profile, also marks transitions between the various parts of gestation. In eutherians, an inflammatory reaction marks the transition from a pre- to a post-implantation phase **(Figure 1.2)**. In marsupials, the inflammatory phase triggered after the first direct fetal-maternal contact is not solved (Griffith et al., 2017), which limits the establishment of a post-implantation system (Moors, 1974). This reaction is followed by an anti-inflammatory phase in eutherians, during which the placenta and fetus have a more symbiotic relation (Wegmann et al., 1993) until parturition (Romero et al., 2006).

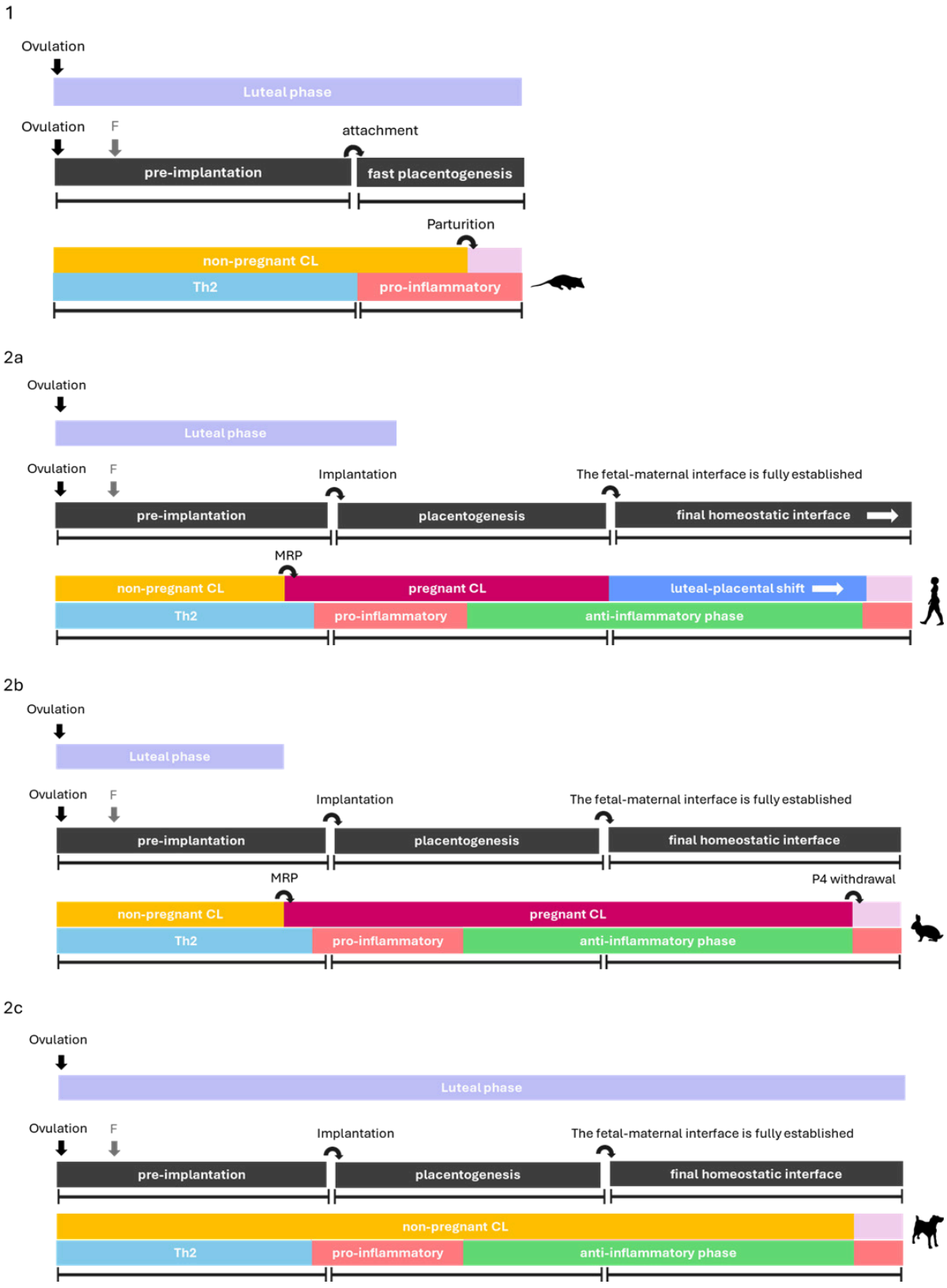

Figure 1. 1) Timeline of pregnancy in the marsupial. 2) Timeline of pregnancy in eutherians. a) human-like b) rabbit-like c) dog-like. F = fertilization. P4 = progesterone. CL = corpus luteum. MRP = maternal recognition of pregnancy. Th2 = T helper type 2 immune response.

BREAKING IT INTO TWO: THE DECOUPLING OF A POST-IMPLANTATION SYSTEM

As mentioned above, in nonmammalian viviparous vertebrates, a pre-implantation system is sufficient to encompass all phases of gestation. This pre-implantation system corresponds to the luteal phase of the ovarian cycle, which is a vertebrate innovation. Tracing the ancestral role of estrogen and progesterone and its acquisition of signaling functions is critical to understanding the evolution of the luteal phase. Both steroids may have originated as a metabolic byproduct of the synthesis and degradation of yolk constituents and cholesterol in the follicular cells (Browning, 1973). In the pre-ovulatory follicle, estrogen was accumulated as a byproduct. As an early function of estrogen was to promote vitellogenesis (Wallace, 1978), its transformation to progesterone in the post-ovulatory follicle would have been a solution to prevent a positive feedback between the accumulation of yolk and estrogen.

The origin of maternal recognition of pregnancy (MRP) was the first step in temporally decoupling the luteal phase from gestation. Inside the ovary, the communication between the oocyte and granulosa cells composing the follicle is essential. Steroid production by granulosa cells relies on the presence of oocytes (Nalbandov, 1972). During maternal recognition of pregnancy, oocyte-follicle communication continues outside the ovary, where the fertilized oocyte communicates the presence of pregnancy to the post-ovulatory follicle.

In **Chapter 1** of this thesis, I argue that pregnancy evolved by a modification of the luteal phase of the ovarian cycle. While showing a gradation in the presence of pregnancy-specific signaling, the luteal phase and pregnancy are interconnected by sharing the same endocrine milieu, namely the progesterone released by the ovary's corpus luteum. These two endocrine and physiological systems, crucial to placental mammalian reproduction, are thus discrete but interrelated. Evolutionarily, these systems are connected by temporal serial homology, achieving the variational independence and individuation that allowed their functional specialization. With the origin of MRP signaling, each system started its evolutionary history and adapted to different selective pressures. Gestation lengths increased in eutherians, likely due to an increasing body mass, while the luteal phase remained the same or got even shorter. The latter can be observed in species with short luteal phases after ovulation. Eutherian mechanisms for MRP are complex and diverse. The absence of a single mechanism to rescue the corpus luteum lifespan across species suggests that they likely evolved independently in different lineages. I explore this diversity and its implications to understand the evolution of gestation length in **Chapter 1**.

The evolution of eutherian pregnancy leads us to a complex interplay between evolutionary coupling and decoupling. On the one hand, pregnancy in placental mammals exhibits a remarkable level of physiological, organizational, and endocrine unity compared to marsupials and non-mammalian viviparous species. By highlighting its relational integration, eutherian pregnancy is an evolved relational novelty (Nuño de la Rosa et al., 2021). On the other hand, the integration of exogenous signals and the transfer of endocrine control from the mother to the fetus, unique to eutherians, also points to an opposite dynamic of increased decoupling. In any preimplantation endocrine system, a high degree of neuroendocrine integration governed by the maternal central nervous system is the rule (Davies & Ryan, 1972). When gestation happens within this system, maternal mechanisms and signaling pathways primarily direct the gestation process. During the evolution of eutherian pregnancy, however, the emergence of a distinct post-implantation system entails a shift to placental gonadotropin signaling, placental steroidogenesis, placental sources of paracrine signaling, and, in cases of invasive placentation, even the replacement of maternal cell types by the placenta.

ON WHAT IS OR IS NOT A REPRODUCTIVE TRAIT

Reproduction is not limited to hormonal changes and the apposition between maternal and fetal tissues during mammalian pregnancy. Physical objects like nests, eggs and shells, and behaviors like maternal care and courtship are also part of a reproductive cycle. This brings us to consider what is and is not a reproductive trait, particularly in those cases where reproductive and non-reproductive functions become blurry. In this thesis, I will emphasize the importance of understanding non-pregnant cycles to study the evolution of pregnancy and its associated innovations, but where does a reproductive cycle, or a reproductive trait, begin and end? What reproductive traits first evolved for other functions and, the other way around, what non-reproductive traits initially selected for a reproductive function are now independent of it?

One compelling boundary case is the link between "reproduction" and "sexuality". These terms have their own historical conception. The term reproduction, reminiscent of the modern notions of production and mechanical reproduction (Jordanova, 1995), replaced the term "generation". The latter, associated with the notions of growth, differentiation and coming into existence, dominated from antiquity until the end of the eighteenth century (Gasking, 1967). Likewise, the concept of sexuality, concerning identities and interactions, did not appear until the eighteenth century (Foucault, 1978). The sometimes ambiguous frontier between reproduction and sexuality uniquely impacts reproductive research. The inevitable connection between these two areas could have even delayed the crystallization and acceptance of reproductive research as a legitimate scientific enterprise (Clarke, 1998). As such, reproductive endocrinology did not emerge as an organized field, with a research agenda and methodology delimited from sexology until the mid-twentieth.

A paradigmatic case of how some traits sit just in between these two fields and the controversies that follow them is the case of the female orgasm. In **Chapter 2**, I delve into the connection between sexuality and reproduction using this unique case study. As part of sexuality, the female orgasm belongs to sexology. Considered as part of a procreative response or behavior, however, this trait falls into reproductive sciences. Reproduction in mammals broadly refers to those body systems involved in conception, gestation, and birth. Abundant research has tried to directly or indirectly link the female orgasm to reproduction and fertility. From this angle, multiple evolutionary accounts have attempted to explain the origin and evolution of the female orgasm. I connect and synthesize these accounts in **Chapter 2**. The female orgasm has been the object of intense debate in evolutionary biology. Interestingly, the different evolutionary views on the female orgasm not only derive from and imply different conceptions of the link between reproduction and sexuality; they also depend on various concepts of homology.

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The shifting role and regulation of the corpus luteum in vertebrate reproduction: a synthetic review

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Abstract

A hallmark of eutherian pregnancy is its dependency on elevated progesterone levels for its entire length. This fact substantially affected the evolution of pregnancy and even the life history of eutherians. Progesterone synthesis by the ovarian corpus luteum, however, long predates all origins of vertebrate viviparity, which requires reconsidering the role of the corpus luteum and progesterone before and beyond placental mammals. Pregnancy originated repeatedly by co-opting the ancestral luteal phase of the ovarian cycle. Therefore, it represents a physiological equivalent and "serial homolog" of the nonpregnant luteal phase. Because of this relationship, we argue that understanding the origin and evolution of pregnancy can substantially benefit from examining the evolution and regulation of the corpus luteum during nonpregnancy. In the first part of this paper, we investigate what is known about the evolution of the corpus luteum and its changing regulation and function across vertebrates, covering both pregnant and nonpregnant cycles wherever possible.

Studying pregnancy in the context of the nonpregnant cycle reveals the key evolutionary innovation that facilitated the characteristically long eutherian pregnancies: the regulatory decoupling of the pregnant and nonpregnant cycle, that is, the evolution of maternal recognition of pregnancy (MRP). Only after the two cycles became separately modifiable, could gestation length increase without deleteriously extending the time to the next fertile phase. In this review, we argue that, assuming selection for body size increase, the origin of MRP led to a punctuated increase of gestation length, which could imply that we overestimate pregnancy length in the eutherian ancestor when using conventional phylogenetic inference methods.

Mechanistically, decoupling between cycles means that the progesterone-dominated phase in pregnancy is extended beyond that of nonpregnancy. In eutherians, this is achieved by extending the lifespan of progesterone-producing corpus luteum, providing additional corpora lutea, extraovarian sources of progesterone, or a combination of these. Alternatively, it can also be accomplished by shortening the nonpregnant cycle. The differences in underlying mechanisms for decoupling among the major eutherian lineages suggest that they originated independently, which

leads us to conclude that eutherian gestation consists of parts that are not homologous across eutherians. Consequently, eutherian gestation *length* is not a homologous trait that can be productively compared across species directly.

As long appreciated, body size explains a major portion of the variation in gestation length in eutherians. Because the prolonged pregnancy is needed to accommodate a large body size, we propose that the individualization of pregnancy from the nonpregnant cycle might have been a key event in the evolution of increased body size.

Introduction

The corpus luteum (CL) is a transient gland resulting from the transformation of the postovulatory ovarian follicle that is vital in vertebrate reproduction. It constitutes the primary source of progesterone, a hormone essential to sustain eutherian pregnancy. The CL's lifespan determines the length of the luteal phase of the ovarian cycle, characterized as a progesterone-dominated phase following an estrogen-dominated follicular phase. The loss of the CL's secretory activity due to regression at the end of each cycle enables the initiation of a new cycle. Therefore, CL's lifespan is a major determining factor of the pace of vertebrate life history. Placental mammals exhibit high interspecific variation in mechanisms of CL lifespan regulation, namely the mechanisms that prolong its lifespan during pregnancy, ensuring a stable progesterone supply, or those that shorten it during the non-pregnant cycle.

Most well-known regulators of CL lifespan are extra-ovarian, deriving from the maternal pituitary, the uterus, or the fetal placenta. Mechanisms that extend CL lifespan by preventing regression ("rescue") after fertilization are known as mechanisms for maternal recognition of pregnancy (MRP). Although these exogenous sources of CL regulation seem unique to Placental mammals, the CL itself is shared among vertebrates. The origin of this gland in early chordates added a phase to the pre-existing ovarian cycle, which at the root of vertebrates consisted of follicle maturation, ovulation, and expelling the eggs from the body. Before the multiple origins of internal fertilization in vertebrates, the CL might have initially enabled the short internal embryo retention associated with internal fertilization. Upon the transition to viviparity, however, it acquired a more distinct supportive role: in non-mammalian vertebrates, despite the independent origins of viviparity, a clear trend points to the CL lifespan of viviparous species being longer than in the oviparous species of each class.

In this review, we identify the key physiological innovations in CL regulation and elaborate on the context and the role that these innovations may have played in the evolution of eutherian pregnancy. To achieve this, we synthesize the literature on CL regulation in a broad vertebrate phylogenetic context, starting with a section on the origin of the CL as an endocrine gland in early vertebrates, followed by review of available knowledge about CL's regulation in nonmammalian and mammalian vertebrates. We pay special attention to the distinct roles of CL in independent origins of vertebrate viviparity. Eutherians are the only taxon in which full gestation is invariably dependent on progesterone, and this requirement has had enormous consequences for the evolution of mammalian traits. Eutherian pregnancy occurs in the time-limited luteal phase of the ovarian cycle, imposing a physiological constraint on gestation length. Decoupling the lengths of the homologous pregnant and non-pregnant cycles (so-called maternal recognition of pregnancy; MRP) thus played a key role in the evolution of gestational length. As we will show, the mechanisms underlying MRP are diverse, suggesting independent origins in major mammalian lineages. This eutherian key innovation furthermore enabled prolonged gestation, likely driven by selection on increased body size, response to which was enabled by MRP.

In short, we show that mammalian gestation is best understood if considered in the context of the female cycle and its evolution, rather than as an independently evolving trait.

1. Early research into the endocrine function of the corpus luteum

The beginnings of corpus luteum research can be traced to Regnier de Graaf (1641–73), who first provided a detailed description, and illustrations of the structures present in the ovaries of pregnant rabbits (Asdell 1928). In 1681, Marcello Malpighi named the structure *corpus luteum* (yellow body, hereafter CL). Gustav Born (1851-1900) first explicitly theorized about its possible function in pregnancy (Simmer 1971), but it wasn't until the early twentieth century when Ludwig Fraenkel demonstrated that rabbits could not maintain pregnancy after removal of all corpora lutea (Fraenkel 1903). Not much later, Leo Loeb showed for the first time that the CL enables implantation by inducing changes in the endometrial tissue (Loeb 1907), and soon after it was demonstrated that luteal extracts could rescue early pregnancy in rabbits after ovariectomy (Allen and Corner 1930). Apart from preparing the endometrium for implantation, it was also discovered that the CL functions to inhibit ovulation. Loeb's interpretation that removing the corpus luteum hastened ovulation in guinea pigs (Loeb 1911) confirmed another long-standing intuition: the gland possessed an anti-ovulatory effect, an action first speculated by John Beard (1858-1924) and Auguste Prenant (1861-1927) (Simmer 1971). Following this idea, Ludwig Haberlandt first demonstrated the suppression of ovulation in mating rabbits after the injection of ovarian extracts (Haberlandt 1922). At the beginning of the 20th century, the research devoted to this gland diversified; with related, if sometimes opposite motivations: from exploring its role in pregnancy, through emphasizing its role on sexual plasticity (Steinach and Kun 1931), to postulating its use in hormonal sterilization and later, contraception (Simmer 1970).

In 1934, several independent groups successfully isolated the gland's active substance, progesterone (Allen and Windersteiner 1934; Butenandt and Westphal 1934, Hartmann and Wettstein 1934), which received its name from a combination of "progestin" and "luteosterone" (Allen et al. 1935). Early comparative research on the endocrine role of the CL briefly challenged the view of CL-progesterone as the mammal-wide pregnancy maintenance hormone. Researchers working on species where the placenta produces progesterone later in gestation, what we know today as the "luteal-placental shift", soon discovered that ovarian progesterone was not equally necessary for pregnancy in all mammalian species, and, among these, the lack of definitive evidence in humans weighed strongly (Reynolds 1939). Csapo and Pulkkinen (1978) reestablished the widespread requirement for progesterone by showing that the corpus luteum was indispensable for early human pregnancy. While they acknowledged species-specific differences in endocrine regulation, such as the presence and timings of a luteal-placental shift, they confirmed the indispensability of the CL in implantation and of progesterone for gestation (Csapo and Pulkkinen 1978).

Various other organs were shown to regulate corpus luteum lifespan and function. Hypophysectomy experiments revealed the supportive function of the anterior pituitary during pregnancy (Pencharz and Long 1931), and further research identified the trophoblast as an important endocrine player supporting CL in several species (Rowson and Moor 1967). Hysterectomies, in contrast, revealed that the uterus is primarily luteolytic, rather than luteotrophic: a signal from the uterus triggers luteal regression in many species (e.g., hysterectomy in nonpregnant guinea pig allows the persistence of the functional CL for up to three months: Loeb 1923, 1927, Rowlands and Short 1959). This interplay between luteolytic and luteotrophic factors was soon recognized to be a decisive factor determining the length of eutherian pregnancy. It was in this context that Short (1969) first defined maternal recognition of pregnancy as those ways in which the embryo first signals its presence to the uterus, which results in a CL of pregnancy protected from luteolytic signals (Short 1969).

2. Evolutionary origin of the CL and its endocrine function

The corpus luteum is embedded within a larger reproductive organ system known as the hypothalamic-pituitary-ovarian axis (HPO axis). The corpus luteum evolved early in the evolution of vertebrates **(Figure 1)**. Corpora lutea are absent in tunicates and cephalochordates (ciona and amphioxus) but present in hagfish ovaries (jawless vertebrates, Agnatha), where they are also likely functional (Gorbman 1983, Powell et al., 2006). Evidence indicates that a CL does not form in the lamprey ovary (Dodd, 1986), which may be attributed to the fact that lampreys die soon after spawning. To understand whether steroidogenic functions originated with the CL itself, we will summarize the evolution of the vertebrate female reproductive system and the context in which the CL was first integrated.

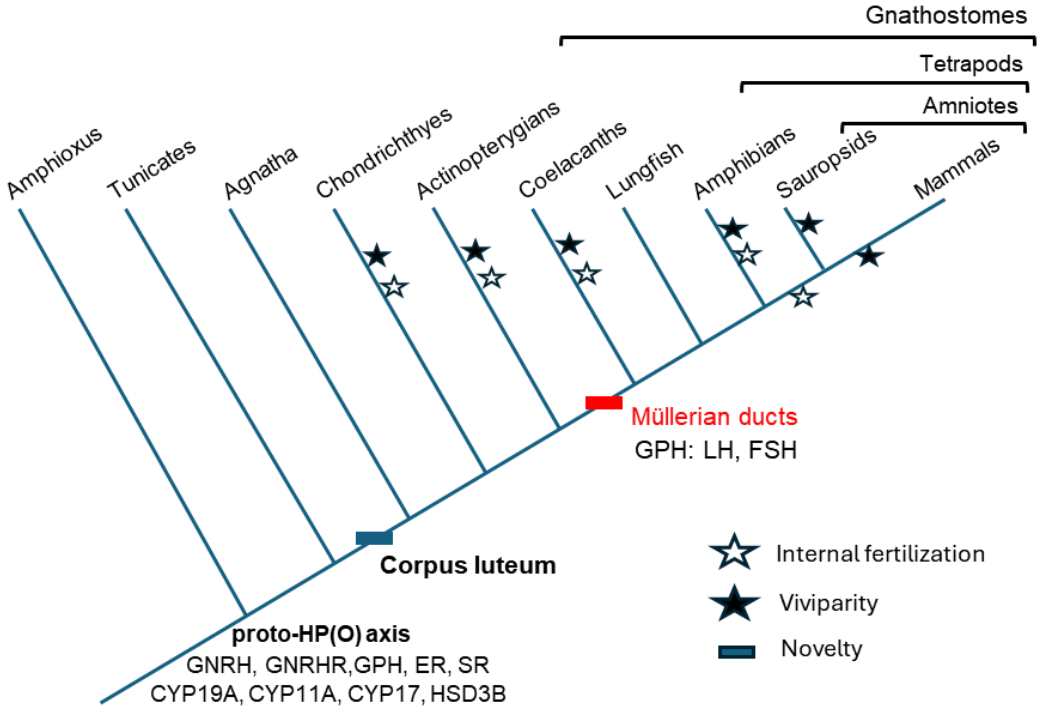

Figure 1. **Early events in vertebrate hypothalamic-pituitary-ovarian (HPO) axis evolution.** Note that the CL predates any of the origins of viviparity. Internal fertilization originated several times independently and was followed in these taxa by the origin of viviparity. Only in Amniotes did viviparity evolve several times on the basis of shared internal fertilization. The asterisks denote that internal fertilization and viviparity originated in these clades, but do imply that all species of the clade share it. CYP11A, CYP17, HSD3B: enzymes in the steroidogenic pathway; GNRH, GNRHR: gonadotropin releasing hormone and its receptor; HPO: hypothalamic-pituitary-ovarian, GPH: gonadotropin-releasing hormone; ER, SR: estrogen and steroid receptor; LH: luteinizing hormone; FSH: follicle stimulating hormone.

2.1. The evolution of the hypothalamic-pituitary-ovarian axis in early vertebrates

Hypothalamic gonadotropin-releasing hormones (GnRH) are the hormones by which eutherian hypothalamus regulates the pituitary release of follicular stimulating hormone (FSH) and luteal hormone (LH). GnRH-like peptides have been detected immunochemically or in genome sequence in a broad range of species, including bivalves and cnidarians (Guilgur et al. 2006). The neurosecreted GnRH family of peptides is thus shared among chordates, and several of its receptors are found in ascidians and amphioxus (Kah et al. 2007, Adams et al. 2003, Chambery et al. 2009, Tello and Sherwood 2009, Roch et al. 2014). The specific amphioxus GnRH, ortholog of mammalian

hypothalamic GnRH-I, is expressed broadly across the nervous system, albeit with 10-fold higher concentration in the anterior part of the body, peaking before the gonadal maturation (Chambery et al. 2009) and influencing gonadal development and function (Fang 1989). GnRH injection in ascidians (*Ciona intestinalis*) induces spawning (Terakado 2001, Adams et al. 2003, Sherwood et al. 2006), and the incubation of rat pituitary with amphioxus GnRH induces a luteinizing hormone (LH) release comparable to that following the incubation with mammalian GnRH, suggesting wide conservation of reproductive functionality (Chambery et al. 2009).

Early chordates lack the pituitary gland, but the Hatschek's pit in amphioxus is considered the homolog of the vertebrate anterior pituitary (Gorbman 1983, Gorbman and Tamarin 1985, Wang et al. 2018; but see Schlosser 2021, pp. 177-180). Hatschek's pit is an oral cavity organ composed of ciliated cells exposed to external stimuli, with likely sensory function (Schlosser 2021). This organ secretes a single heterodimeric glycoprotein hormone (thyrostimulin) and a cytokine that may be related to prolactin (Li et al. 2014). The reproductive role of Hatschek's pit is supported by the fact that its damage prevents gonadal development and ovulation in amphioxus (*Brachiostoma belcheri*; Fang et al. 2001). A homolog of the pituitary gland in ascidians remains unclear, but an organ called the neural gland complex has been proposed (Ruppert 1990, Kano 2010).

The amphioxus ovarian follicular cells show morphological signs of steroidogenesis (round mitochondria, large lipid droplets, large Golgi complex, ER; Welsch and Fang 1997), and genomic alignment as well as the functional studies confirm the presence of the major steroidogenic enzymes (*CYP19A*, *CYP11A*, *CYP17*, *HSD3B*) (Kubokawa et al. 2010, Markov et al., 2017, Mizuta and Kubokawa 2007, Callard et al. 2011; Zhang et al., 1984). However, the production of sex steroids does not necessitate signaling via their cognate receptors. We will address their possible early functions below.

The most phylogenetically distant, fully functional vertebrate hypothalamic-pituitary-ovarian axis likely occurs in hagfishes (Agnatha), which already have a two-lobed pituitary. Immunoreactive gonadotropin releasing hormone GnRH has been identified in the Atlantic hagfish (*Myxine glutinosa),* where it is associated with the seasonal reproductive state (Kavanaugh et al. 2005, Powell et al. 2004). Removal of pituitary in the inshore hagfish (*Eptatretus burgeri*) affects gonadal development, confirming pituitary regulation of ovarian function (Patzner 1977). Agnathans have only one pituitary glycoprotein hormone, GPH (homologous to the ancestral thyrostimulin: Uchida et al. 2013). This heterodimeric hormone has diversified by duplications of the alpha and beta subunits in the stem lineage of jawed vertebrates (Gnathostomes). Thereby the ancestral GPH, which in cephalochordates and agnathans was already involved in gonadal development and spawning, gave rise to the family of heterodimeric pituitary gonadotroph hormones (including thyroid stimulating hormone, luteinizing hormone, and follicle stimulating hormone; Kawauchi and Sower 2006).

Ovarian sex steroids also diversified early in vertebrate lineage (Markov et al. 2009). The order in which ovarian steroid signaling functions originated is determined by the evolutionary expansion of their receptors (Baker 2003). The ancestral estrogen receptor (ER) resulted from a gene duplication of the ancestral steroid receptor (SR) in basal chordates, as both are present in amphioxus **(Figure 1)**. Ovarian estradiol is reported to signal back to the hypothalamus-pituitary level, which implies that estrogen signaling is in place in agnathans (Nozaki 2013). The ancestral progesterone receptor (PR), on the other hand, arose in a 3-ketosteroid receptor gene duplication prior to the divergence of agnathans (Baker 2019), which would mark the earliest possible origin of progesterone signaling function. Finally, early gnathostome genome duplication resulted in sub-functionalization of the ancestral ER into ERa and ERb, shared between cartilaginous fishes and mammals (Baker 2019).

Taken together, comparative evidence suggests that the predecessors of all segments of the hypothalamic-pituitary ovarian axis (GnRH, gonadotrophs and steroids) were present and had reproductive functions in stem chordates, predating the origin of the corpus luteum. However, these

reproductive functions were likely less entrenched and specific. Broad expression of GnRH across tissues in amphioxus (Tsai and Zhang 2008, Schlosser 2021) suggests that GnRH in early chordates might have served a more general regulatory function than female reproduction and later became reproduction-specific. Photoperiod-sensing, including the synchronization of spawning with environmental light conditions, could be one such function. Indeed, spawning in tunicate *Ciona* is regulated by the photoperiod and can be induced by GnRH (Sherwood et al. 2006), suggesting that the latter mediates the reproductive effects of seasonal light changes (as reported in many mammals).

Upon the origin of the hypothalamic-pituitary-ovarian axis, the integration of a further chemosensory input from a pituitary-like Hatschek's pit with a photosensor controlling GnRH expression would have resulted in the coupling of spawning to increasingly specific conditions, leading to the reproductive specialization of vertebrate hypothalamic-pituitary-ovarian axis. The reproduction-specificity of this signaling axis became further entrenched following gnathostome-specific genome duplication, which resulted in diversification of pituitary glycoprotein hormones and steroid hormone receptors. Moreover, this diversification happened in jawed vertebrates concurrently with the appearance of the reproductive tract (Müllerian ducts and their derivatives), which were eventually integrated as targets of the hypothalamic-pituitary-ovarian signals. This ensuing entrenched complexity of a network of interdependent processes, may have, in turn, prevented subsequent substantial evolutionary change of the network, resulting in a structurally conserved gnathostome hypothalamic-pituitary-ovarian axis.

2.2. The origin of the progesterone-rich luteal phase

The development of the ovarian corpus luteum from the ovulated follicle initiates the luteal phase. The origin of the luteal phase introduced a switch in ovarian hormone production from estrogen of the maturing follicle to progesterone of the corpus luteum.

As shown, the ability to synthesize ovarian sex steroids and the origin of some steroid receptors predated the origin of the corpus luteum in stem jawed vertebrates **(Figure 1)**. Browning (1973) proposed that ovarian steroid synthesis may have evolved to metabolize and remove the surplus cholesterol of the yolk (Browning 1973). According to this hypothesis, the ovarian steroids are a byproduct, initially lacking a signaling function. Indeed, the enzymes involved in steroidogenesis most likely evolved from xenobiotic catabolic pathways (Markov et al. 2009). Furthermore, their biosynthesis from cholesterol involves addition of hydroxyl groups, increasing their water solubility and thus making the products easier to transport passively and dispose of, further supporting Browning's scenario. Given the appropriate receptors transducing the signal to the nucleus, precisely this hydrophilic nature might have enabled the co-option of steroids into the role of long-distance signaling molecules. The most ancient metabolic byproduct of this pathway likely was estrogen (Browning 1973), possibly because of its greater stability than the 3-keto steroids. Progesterone is an intermediate of estrogen biosynthesis; therefore, its synthesis came "for free" with the presence of estrogen, and predated its co-option into a signaling role (Thornton 2001, 2003).

Already in the jawless hagfish, estrogen enhances yolk formation (Nozaki 2013) in the liver, and consequently lipid yolk accumulation in the maturing oocyte. Metabolizing yolk cholesterol to estrogen would thus be counterproductive, given that it is at the same time pro-vitellogenic. In this situation, it may have become advantageous to reduce the conversion of cholesterol into estrogen, and instead accumulate progesterone-related molecules in the post-ovulatory follicle. It is thus conceivable that postovulatory progesterone accumulation might have evolved prior to progesterone's PR-mediated functions and could have constituted an alternative cholesterol metabolite. In the same vein, decreased conversion into estrogen would have provided a way to delay vitellogenesis and impose cycle control. Early jawed vertebrates already possess a form of PR of uncertain functional

role, but it is not clear to us when first progesterone signaling evolves. The above scenario could explain co-options of progesterone in the reproductive system, even prior to its signaling role.

In summary, the corpus luteum (CL) originated in the stem lineage of vertebrates, after the precursor of the hypothalamic-pituitary-ovarian axis had already been established. The origin of CL predates the diversification of the pituitary gonadotropic hormone family, as well as the origin of the Müllerian duct and likely also the origin of progesterone's signaling functions. Consequently, the CL originated in the context of external fertilization and external embryo development, in the context very different from the roles in mammalian reproduction. If the CL's ability to accumulate progesterone derived from the need to replace estrogen in yolk lipid disposal (Browning 1973), its ancestral lifespan was presumably positively correlated with the amount of yolk in the follicle, and therefore intrinsic to the ovary, rather than regulated by the extra-ovarian mechanisms. These amounts probably increased in early vertebrates with yolk-rich eggs and the origin of the liver. Regulation of progesterone conversion into estrogen could have provided a way to regulate the length of CL lifespan and the dynamics of the cycle, by generating the estrogen-progesterone switch.

It is an open question how precisely progesterone was co-opted into its reproductive and specifically into its signaling roles. However it happened, the origin of the progestogenic CL generated a context in which many key functions could evolve, eventually leading to new reproductive modes with different degrees of maternal internal support. With the origin of Müllerian ducts as an oviductal structure in gnathostomes **(Figure 1)**, the temporal coordination of the ovarian cycle with the activity of the reproductive duct likely was of considerable advantage. Further functional differentiation of the CL signaling thus likely occurred in coevolution with an increasing regionalization of the Müllerian duct, allowing the latter to specifically respond to ovarian steroid dynamics by generating secretions and controlled muscular contractions. This initial coordination between the ovary and the reproductive tract became ever more complex with the evolution of internal fertilization, when the simple expelling of eggs was replaced by prolonged internal embryonic development and the production of histotroph, albumen and eggshell. To better understand the origin of the highly derived mammalian CL functions, in the next section, we first review non-mammalian lineages.

3. CL endocrine function and its regulation in non-mammalian vertebrates

Vertebrates display a high diversity of reproductive modes, including multiple origins of internal fertilization, ovoviviparity (egg retention), and viviparity. In this section, we summarize what is known about the role of the corpus luteum and progesterone in non-mammalian vertebrates. We will avoid teleosts despite their reproductive diversity, as it has undergone additional lineage-specific genome duplications and extensive modifications of the female reproductive tract anatomy, complicating the comparison with the remaining jawed vertebrates. The comparative morphology of the non-mammalian corpus luteum is well described, and steroidogenesis is confirmed. Less understood are the mechanisms of its formation, maintenance and its functional significance.

3.1 Internal fertilization and viviparity increase the cost of sterile cycle

The sexual asymmetry of investment in post-gametogenic reproductive processes increased repeatedly in jawed vertebrates. Relative to the ancestrally symmetrical situation between males and females, consisting of expulsion of gametes, female investment increases with the origins of internal fertilization and then again with viviparity. Internal fertilization arose independently in lineages of cartilaginous fishes (sharks and rays), teleosts, amphibians and in amniotes. Increased female internalization of the reproductive events not only intensified the maternal investment in reproduction, but also augmented the cost of an unfertilized cycle. In species with spontaneous ovulation, the

association between ovulation and fertilization is not guaranteed. Unfertilized cycles incur the loss of eggs and delay in reproduction, but also the loss of investment in physiological preparation for fertilization and/or pregnancy. This is less prominent in species with induced ovulation but can occur if the inducing mechanism is not the mating itself, but e.g., photoperiod. Consequently, the species with potential for an unfertilized (or sterile) cycle show two distinct contexts: the luteal phase of the fertile cycle (passing or incubating a fertilized egg and a fetus) and that of the sterile cycle (unfertilized egg or nonpregnant luteal phase). Although the sterile cycle may not often occur in the wild (Conaway 1971), its length nevertheless indicates a periodicity of the corpus luteum lifespan that may constrain or drive the evolution of gestation length and breeding seasonality.

Internal fertilization is necessary for the origin of viviparity. Remarkably, viviparity arose independently in at least some species of every major clade capable of internal fertilization, except for birds (Blackburn 2015): teleosts, chondrichthyes, amphibians, reptiles, and therian mammals, but only in the latter it involves stabilized long-term implantation and is present in the whole clade. The length of CL lifespan coevolved with the reproductive mode: most instances of the evolution of viviparity involve an extension of CL persistence, although corpus luteum is not required for the entire pregnancy in most nonmammalian vertebrates, as shown below.

3.2 Cartilaginous fishes (Chondrichthyans)

These most basally branching gnathostomes have a functional HPO axis consisting of hypothalamic GnRH and two anterior pituitary gonadotropins, which regulate follicular development and ovarian estradiol levels in oviparous as well as viviparous species (Awruch 2013). Many studies note the variable steroid hormone dynamics and its consequence for temporal separation of the follicular and luteal phases. Estrogen increases during folliculogenesis and is vitellogenic in this group. When estrogen levels remain elevated after ovulation, folliculogenesis continues, and new mature preovulatory follicles appear soon after oviposition. If, in contrast, estrogen peaks shortly before ovulation and decreases thereafter, folliculogenesis is suppressed after ovulation. The first scenario characterizes many oviparous and non-seasonal breeders (e.g., the Little skate, *Leucoraja erinacea,* Koob and Callard 1999; or the Draughtboard shark, *C. laticeps*, Awruch et al. 2008). The second scenario applies to viviparous species such as the aplacental Atlantic stingray (*Dasyatis subina*; Tricas et al. 2000). This suggests that in viviparity, ovarian progesterone generates a new phase of the ovarian cycle, a distinct non-folliculogenic luteal phase, by suppressing either vitellogenesis or folliculogenesis. Indeed it has been shown that hepatic vitellogenesis is less sensitive to estrogen during pregnancy in this group (Callard et al. 1990).

Luteal progesterone is restricted to the reproductive season and low or absent in absence of ovarian cycle (Lutton 2011). In oviparous species, progesterone peaks shortly before ovulation (e.g., in the Little skate, *L. erinacea*; Koob and Callard 1999), decreases before egg encapsulation, and is low during egg passage and oviposition. It thus could have a role during the periovulatory phase (Koob et al. 1986). In viviparous species, the decrease of progesterone levels is postponed, and progesterone remains high for at least the first part of pregnancy and in some species until parturition (e.g., the Round stingray, *U. halleri*; Mull et al. 2010). Luteal progesterone thus appears to be associated with egg retention and pregnancy in elasmobranchs, possibly complementing lecithotrophic provisioning by enhancing uterine histotroph secretion, or inhibiting myometrial contractions. We found no report on CL lifespan in sterile cycles and no indication of embryonic or placental control of luteal phase in this group (but see Hamlett 1999, Callard and Koob 1993). The data from the viviparous dogfish shark *M.canis* suggest that while the pituitary is necessary for folliculogenesis, it is not required for CL formation or maintenance (Dodd 1983), implying that the lifespan of elasmobranch CL may be inherently determined, potentially by the amount of cholesterol remaining in the CL, possibly proportional to the amount of yolk.

3.3 Amphibians

The CL has also been described in oviparous and viviparous amphibians. While most anurans have external fertilization, most salamanders and all caecilians fertilize internally, and many of them evolved viviparity (Wake 1980, Wake and Dickie 1998). One anuran exception are the toads of the genus *Nectophrynoides*. The Nimba toad (*N. occidentalis*) ovulates yolk-poor eggs, and the internally fertilized embryos are nourished by extensive secretions of the female reproductive tract during a long pregnancy, resulting in the birth of metamorphosed young (Xavier 1978, reviewed in Sandberger-Loua et al. 2017). This species is an example of an independently evolved prolonged luteal phase involving the same upstream factors but different downstream mechanisms and consequences. Nimba toad has distinct estrogen-rich follicular and progesterone-dominated luteal phases, accompanied by extensive remodeling of the gestational part of the reproductive tract. The progesterone-secreting CLs persist for the entire gestation length. Remarkably, this species manifests pseudopregnancy without mating, maintaining extensive remodeling of the reproductive tract. Xavier (1978) demonstrated that progesterone is necessary for early pregnancy but is not crucial for maintaining gestation thereafter. The effect of initially high levels of progesterone, however, is to slow embryonic development in early pregnancy, which occurs during a dry season with toads burrowed and inactive. After emerging from the ground, decreasing progesterone levels accelerate development, and progesterone becomes dispensable. In addition to growth regulation, progesterone is crucial for reproductive tract remodeling, particularly in primiparous females. The female tract supports the CL in early pregnancy, as hysterectomy before emergence from the ground leads to luteolysis. There is also ovary-pituitary feedback, as ovarian signaling affects the cell composition of the pituitary during vitellogenesis and early gestation.

Viviparity originated multiple times in salamanders, yet it is rare and variable in extent, from birthing aquatic larvae to fully metamorphosed young. It mainly entails less yolk than ovoviviparous populations (even within the same species; Buckley et al. 2007), and additional provisioning by maternal secretions. Steroidogenic corpora lutea are present in salamanders, however, the plasma progesterone, as estrogen, stays relatively low, whereas testosterone levels increase after ovulation. High progesterone is limited to the ovary during pregnancy, but is low in plasma. Progesterone has been associated with vitellogenesis and epithelial and vascular remodeling of the reproductive tract to accommodate pregnancy, but is not required to maintain pregnancy.

The amphibian clade with most widespread internal fertilization are cecilians (Gymnophiona), which also evolved aplacental viviparity. In well-studied viviparous caecilians, ovulation is triggered by endogenous and exogenous cues (e.g., *Typhlonectes compressicauda*, Brun et al. 2020). The progesterone-producing CL of fertilized females is maintained throughout pregnancy, degenerating only at parturition. The physiological relevance of progesterone in gestation is unknown but it could enhance reproductive tract secretions after hatching. The fetuses hatch from the egg membrane after the abundant yolk had been absorbed, and it has been reported that fetal teeth are used to ingest endometrial secretions (Wake 1977a, 1977b). Wake (1977a) described the distinct remodeling of the reproductive tract in viviparous caecilians during pregnancy, with increased epithelial proliferation and the forming of gland-like secretory pockets. It is unclear whether this remodeling is induced by fetal presence and thus limited to pregnancy or is maternally induced, in which case it would be present in unfertilized females.

Overall, the corpus luteum of oviparous amphibian species disintegrates soon after ovulation, whereas it is maintained longer in the fertilized ovoviviparous and viviparous amphibians (with the exemptions of salamanders), in some viviparous species up to complete gestation. A shared characteristic of this group is the role of progesterone in remodeling of the reproductive tract for the internal part of development in ovoviviparity and viviparity. Other roles of progesterone are

species-specific, such as slowing the embryonal development of Nimba toad, or possibly epithelial hyperplasia in caecilians. Together, this suggests that the evolution of ovoviviparity and viviparity in amphibians is associated with a prolonged persistence of postovulatory corpus luteum. Whether this CL prolongation is due to maternal (as in Nimba toad) or fetal signals remains an open question.

3.4 Reptiles and Birds

Internal fertilization is a shared character of amniotes, resulting in at least a minimal degree of internal embryo support, even in egg-laying species. The CL is the main source of progesterone in most reptilian species (Bragdon 1952, Highfill and Mead 1975), and its lifespan correlates with the length of embryo retention in the female tract, across species. In oviparous species, progesterone levels decrease soon after ovulation, but the CL is maintained until egg-laying. In fact, the surgical removal of the corpora lutea causes premature oviposition in all oviparous lizard species examined (Guillette 1987, Jones and Baxter 1991), implying that some other product of the CL rather than progesterone is required for egg retention.

The corpus luteum of viviparous lizards persists for a variable portion of gestational time (Cieslak 1954, Weekes 1934), and progesterone may remain elevated during this time. However, the structural degradation of the CL is observable well before parturition, even in those species in which CL is histologically recognizable until after parturition (Weeks 1934, Bennett and Jones 2002). Ovary removal (ovariectomy) does not cause a shortening of internal development in viviparous or ovoviviparous lizards or snakes (Amoroso and Finn 1962), except if performed very early in pregnancy, in which case it can impact either pregnancy length or parturition (Clausen 1940, Fraenkel et al. 1940, Yaron 1972, Guillette 1987). Intriguingly, squamate reptiles (but not turtles and crocodiles) use a single pituitary gonadotropin in ovarian regulation (Licht 1983). While the orthologs of mammalian FSH and LH -specific ß-chains have been found in the genome, it is unclear whether they produce one or two different proteins (Bluhm et al. 2004). Pituitary removal followed by injection of heterologous mammalian FSH or LH hormones did not affect reproduction in Common garter snake (*Thamnophis sirtalis)* or Common lizard *(Lacerta vivipara;* reviewed in Norris and Jones 2012, p.263). Bragdon (1951) found that pituitary removal in Common watersnake (*Natrix sipedon)* and Common garter snake did not interrupt pregnancy at any stage (but see Clausen 1940), including the first week after ovulation. In contrast, ovariectomies prior to the second trimester result in interruption of pregnancy (Bragdon 1951), implying that the ovary (likely CL) is crucial for the first trimester of pregnancy. In short, pituitary hormones are required for squamate folliculogenesis but not for luteal development and maintenance or any other pregnancy support , whereas ovary is required at least for the first part of pregnancy (Yaron 1972).

The avian post-ovulatory follicle regresses rapidly after ovulation, without proliferation, luteinization or hypertrophy. The name avian corpus luteum thus appears a misnomer (Davis 1942, Payne 1966). However, the postovulatory follicle maintains a function in determining the interval between ovulations: removing the post-ovulatory follicle delays egg-laying, but the underlying mechanism is unclear (Rothchild and Fraps 1944).

In summary, in most non mammalian clades studied, the CL lifespan in the fertile cycle of (ovo)viviparous species is longer than that of the closely related oviparous species (Gemmell 1995). Without information on the presence and length of sterile cycles we cannot address the question whether longer luteal phase of viviparous species is paired with longer sterile cycle, or prolonged independently from it, implying a mechanism for the “maternal recognition of pregnancy” (see below). One of the first functions of progesterone likely entailed inhibiting vitellogenesis in the liver and preparing the reproductive tract for egg formation or internal development. Different degrees of vitellogenesis inhibition were observed during pregnancy in viviparous elasmobranchs (Teshima and Mizue 1972, Hisaw and Abramowitz 1973, Tsang and Callard 1987) and are also encountered in

amphibians and reptiles (Callard et al. 1985). Inhibition of vitellogenesis prevents the subsequent ovarian follicular growth and ovulation during a fertile cycle, thus spacing the follicular phases and individuating the luteal phase - aspects of the cycle widely shared among nonmammals and mammals alike. In addition, progesterone obtained functions in the early luteal remodeling of the reproductive tract, such as preparing the reproductive tract or enhancing eggshell production or nutritive secretions. While the role in the reproductive tract is generally widely present, its exact realization, i.e., which aspects of internal development are enhanced, appears species-specific. We suggest that these latter functions explain the variation observed in progesterone dependence after performing ovariectomies at different gestation times in non-mammalian species. Overall, the increased progesterone levels throughout pregnancy are not a shared requirement of non mammalian viviparity.

The origin of the CL prior to internal fertilization and internal development, and the fact that only the formation of CL but not its lifespan, is regulated by ovary-external maternal signals in at least the early branching vertebrate lineages, together suggest that the lifespan of CL was ancestrally inherent to the ovary (see below). The responsiveness to signals from the endometrium and the trophoblast is thus likely a derived feature of CL, as already suggested by Rothchild (1981). The evolution of the role of the pituitary in *maintaining* the steroidogenic postovulatory CL or *extending* its lifespan beyond the length of the luteal length in the sterile cycle, is not yet understood.

4. CL endocrine function and its regulation in mammals

Mammalian pregnancy originated independently of non mammalian occurrences. Within theria, ancestral marsupial pregnancy is superficial and short, whereas the ancestral pregnancy of placental mammals (eutherians) is characterized by implantation and a highly invasive placentation. In the eutherian mammals, elevated progesterone levels are necessary for the entire pregnancy length. Thus, only in this group, progesterone has a consistent role also in pregnancy *maintenance*, which may be related to the unique presence of invasive embryo implantation, or the lack of yolk (Rothchild 2003).

In this section, we will consider this novel, fully progesterone-dependent maternal-fetal relationship as a driving factor for the evolution of mammalian CL regulation. In mammals, the lifespan of the CL often differs between pregnancy (CL of pregnancy, pCL) and the luteal phase of sterile cycle (CL of nonpregnancy, npCL). This decoupling of CL regulation in pregnancy from the sterile cycle could have resulted from a selective pressure to prolong pregnancy, while maintaining a short sterile cycle. A long sterile CL lifespan incurs a long interval to the next ovulation and thus lower reproduction rate, and would likely be disadvantageous in most conditions. Exemptions are the species in which the natural interval between ovulations is particularly long, such as in seasonal breeding, or which very rarely encounter sterile cycles, such as species with copulation-induced ovulation. In species where the ovulations occur regularly in shorter intervals, mechanisms to shorten the CL lifespan in sterile cycle, or prolong it only when fertilized, are expected to increase reproductive output and thus fitness. Understanding the regulation of pCL thus requires comparison of the CL of pregnancy (pCL) to that of the nonpregnancy (npCL). We have included the latter whenever available, it is however rarely reported, and probably rarely realized in the wild even where it is physiologically possible. Often the whole nonpregnant estrous cycle can serve for orientation instead.

In most eutherians where known, the sterile cycle is shorter than the length of intrauterine fetal development. In contrast, pregnancy occurs within the length of the sterile cycle in most marsupials. For example, in gray-tailed opossum (*Monodelphis domestica*) pregnancy occurs within the length of sterile luteal phase. This marsupial condition suggests that the pCL and npCL lifespans diverged early in the evolution of eutherian pregnancy. In some eutherian species (e.g., the dog), the sterile and pregnant luteal phase are of equal length, but this is an exception and not the rule, as will be discussed below. In such species, only a few changes are associated with the pregnant cycle. In canids, for

example, the latter includes a pregnancy-specific prolactin elevation and delayed oocyte maturation (Papa and Mariusz 2020).

Eutherians manifest a wide range of regulatory mechanisms for either prolonging the progesterone secretion by pCL, complementing it from other sources, or shortening the npCL by luteolytic factors, thus accelerating the transition into the next, potentially fertile cycle. Most of the known eutherian luteotrophic or luteolytic mechanisms are extrinsic to the ovary. Consistent evidence suggests that ancestral CL lifespan regulation however was intrinsic to the ovary. To understand how the intrinsic CL regulation may have become modified to decouple pCL and npCL, we will next review the intrinsic regulation of this endocrine gland.

4.1. Ovary-intrinsic regulation of CL lifespan

In contrast to the oscillatory activity of other endocrine glands that are regulated by the negative feedback from the pituitary (e.g., the thyroid), the CL lifespan has an intrinsic regulatory component (Rothchild 1981). The primary endogenous mechanism of CL lifespan control consists of a balance between steroidogenesis and the production of reactive oxygen species (ROS). The latter result primarily from the mitochondrial steroidogenesis as well as from the local cellular respiration (Bose et al 2002, Stocco 2000 and 2001, Chapman et al. 2005). The CL undergoes structural and functional regression when the balance of these processes tilts towards ROS accumulation. ROS induce apoptotic cell death (Kato et al. 1997, Behrman et al. 2001, Garrel et al. 2007) and interfere at several levels with progesterone synthesis; either by impairing LH signaling, inhibiting the translocation of cholesterol to the mitochondria, or interrupting the activity of the rate-limiting steroidogenic enzyme CYP11A1 (Behrman et al. 2001). Balancing this destructive process, ROS production is inhibited by steroid hormones and luteal antioxidants, mostly superoxide dismutases and small antioxidants (Hanukoglu 2006). While progesterone directly counteracts ROS production, luteotrophins increase antioxidants, therefore decreasing ROS activity. Given this endogenous lifespan control mechanism, luteotrophic or luteolytic factors converge on modifying the production or activity of ROS and that of antioxidants (Al-Gubory et al. 2005, Sugino 2006).

Rothschild's suggestion that the CL lifespan is determined by the intrinsic balance of progesterone and prostaglandin production complements the evolutionary reconstruction of Browning (1973), according to which prostaglandins first served follicle's fast post-ovulatory regression, and the origin of progesterone signaling allowed a scenario in which this regression could be delayed. Prostaglandins are important modifiers of the lifespan of CL. Prostaglandins F2α and E2 exert autocrine, paracrine, and endocrine effects that depend on the receptors they bind to (Narumiya et al. 1999; Narumiya and FitzGerald 2001). By binding to FP and EP1 receptors, prostaglandin PGF2α, a common luteolytic factor, activates the phospholipase C-protein kinase pathway, decreasing the translation of steroidogenic enzymes and steroidogenic acute regulatory protein (StAR) (Sandhoff and McLean 1996, Niswender et al. 2000). In parallel, PGF2α facilitates ROS accumulation by decreasing the expression of ROS scavenger proteins (Foyouzi et al. 2005). ROS feeds back to enhance PGF2α's abundance, leading to an accelerating process of luteolysis. The locally produced O_2, NO, and H_2O_2 activate the enzymes involved in the synthesis of prostaglandins, further enhancing luteal production of PGF2α (Arosh et al. 2004). In contrast to luteolytic effects of PGF2α, the prostaglandin PGE2, by binding to EP2 and EP4 receptors, activates protein kinase A (PKA), inducing the transcription of genes involved in steroidogenesis, angiogenesis, and cell survival (Ziecik et al. 2018). PKA is also phosphorylated in response to external luteotrophic factors such as LH, indicating that endogenous and exogenous factors have synergistic effects. The decrease in the PGE2:PGF2α ratio restricts luteal blood flow and inhibits steroidogenesis, as well as decreases StAR expression while increases prostaglandin G/H synthase and the release of oxytocin by the CL (Milvae et al. 1996, 2000). The

PGE2 to PGF2α ratio is thus essential to CL's lifespan (Blitek and Ziecik 2005; Breuiller-Fouché et al. 2010).

Although the CL lifespan results from a balance between luteotrophic and luteolytic factors and that, for almost all mammalian species studied so far, ovary-intrinsic or extrinsic PGF2α is the primary luteolysin, the exact mechanisms by which prostaglandin F2α initiates luteal regression are not entirely known. PGF2α loses its luteolytic effect when acting upon isolated steroidogenic cells, revealing that it likely requires further factors (Hansel et al. 1991). One example of a mechanism of action of PGF2α is the activation of endothelin 1 (ET-1), which binds to receptors in the steroidogenic cells and decreases LH stimulation of progesterone and blood supply to the CL (Milvae 2000). The importance of angiogenesis and the role of luteal microvasculature have been extensively revisited (Reynolds et al. 2000, Davis et al. 2003, Tamanini and De Ambrogi 2004). Follicle luteinization requires the action of angiogenic factors that allow LDL cholesterol to reach the luteal cells and be converted into progesterone. Endothelial cells, possibly via secretion of the luteotrophic prostaglandin PGI2, can enhance P4 secretion in the luteal cells (Grish et al. 1995); endothelial factors also play a role in luteal maintenance, as well as regression.

Besides the positive feedback between PGF2α and ROS production, immune cells and cytokines are also involved in CL cell death. In turn, the expression of proinflammatory cytokines by the immune cells recruited into the CL from the systemic circulation constitutes an early signal of CL regression. The luteotrophic and luteolytic nature of immune factors depends on the luteal environment, particularly on the absence of immunosuppressive factors such as progesterone (Walusimbi and Pate 2013). Macrophages likely play a crucial role in the resolution of the luteolytic process and avoidance of an inflammatory response (Pate and Keyes 2001), whereas T lymphocytes enhance progesterone production in the CL of several species (Emi et al. 1991, Hughes et al. 1990 and 1991). On the other hand, progesterone inhibits lymphocyte proliferation and interferes with the cytokine action on the luteal cells (reviewed by Siiteri and Stities 1982, Grossmanm 1984, Kelly 1994). These protective effects weaken as progesterone concentration declines, which eventually precipitates CL regression (extensively reviewed by Pate and Keyes 2001, Davis and Rueda 2002, Cannon and Pate 2003, Bornstein et al. 2004).

Much of the research on luteolysis is conducted on mammals, yet it is likely these mechanisms apply broader. ROS production is a common outcome of ubiquitous cellular biochemical processes, such as steroidogenesis, respiration or hydroxylation, and is conserved among vertebrates. The origin of key enzyme involved in prostaglandin biosynthesis, the cyclooxygenase (in vertebrates isozymes COX-1 and COX-2) predates vertebrates (Knight et al. 1999, Kolijak et al. 2001, Valmsen et al. 2001). Similarly, prostaglandin F has been associated with spawning and hatching processes in marine invertebrates (Ruggeri and Thoroughgood 1985), confirming broad phylogenetic distribution. It is thus likely that the ROS and progesterone-mediated, intrinsic CL lifespan control and the luteolytic mechanism are also shared widely among vertebrates.

Finally, part of the intrinsic mechanisms for CL maintenance could derive from the mechanisms underlying the resolution of inflammation after follicular injury of ovulation (Espey et al. 2004). Inflammation precipitates angiogenesis, resulting in the infiltration of leukocytes into the preovulatory follicle and the development of new vessels during CL formation. Although the immune dynamics and cell composition over the ovarian cycle are complex, it has been suggested that the immune response shifts toward a response akin to wound repair and tissue regeneration after infection or injury, in the luteal phase (Bouman et al. 2001, Faas et al. 2000).

The evolution of eutherian non-ovarian sources of CL regulation could have been connected to another significant innovation of eutherian reproductive biology, the decidual cell. The decidual cell and the functional CL are transient structural entities necessary for successful embryo implantation that result from a transformation of the stress response. The emergence of decidualization might have

promoted the new signals for prolonging the CL cycle, as the evolutionary origin of the decidual cell was probably related to a stress reaction at the molecular and cellular levels (Erkenbrack et al. 2018, Wagner et al. 2019). Many signals for MRP, like CG, PGE2, or PRL, have anti-apoptotic roles on both the CL and stromal fibroblasts, and early embryonic signals work through similar mechanisms in the luteal and decidual cells to prevent cell death and resolve the stress response. Furthermore, luteal progesterone has protective anti-ROS effects on both the CL and the decidual cell. For instance, progesterone increases superoxide dismutase activity in the endometrium, decreasing ROS and prostaglandin F2α (Sugino et al. 1996, Sugino et al. 2000, Sugino 2006). Finally, many of the molecules released by the blastocyst to ensure uterine receptivity, such as PGE2, play luteotrophic roles as well (Psychoyos and Gravanis 1995).

In summary, the intrinsic regulation of CL lifespan thus results primarily from a balance between steroidogenesis, ROS and prostaglandin production, and luteotrophic and luteolytic ovary-extrinsic signals affect CL lifespan by modifying this balance. CL's lifespan susceptibility to exogenous regulation by hormones of various origins, namely the maternal pituitary, the endometrium, or the placenta, makes the CL a unique regulatory hub that integrates input from multiple sources (Stormshak et al. 1987). *When* this susceptibility first evolved, remains to be elucidated.

4.2. Ovary-extrinsic regulation of CL lifespan

As previously mentioned, maternal recognition of pregnancy (MRP) is conventionally defined as the mechanism that prolongs the luteal progesterone production in pregnancy (Short 1969) beyond that of nonpregnancy, i.e., “rescues” the CL from luteolysis. MRP occurs soon after blastocyst formation in eutherians, with the trophoblast inducing changes in CL as well as in the uterine endometrium. Accordingly, most accounts consider MRP a unique eutherian trait (Flint et al. 1990). Recently, authors have applied the MRP concept more broadly to designate any alteration in the maternal physiology during pregnancy that distinguishes it from the nonpregnant condition. For example, this broader notion, applicable to marsupials, may encompass the difference between the pregnant and non-pregnant uterus, also referred to as endometrial recognition of pregnancy (Laird et al. 2017, Griffith et al. 2019).

A CL “rescue” function of placental signals does not seem to be present in marsupials. Although blastocyst-mediated CL reactivation following hypophysectomy occurs in marsupial embryonic diapause (Tyndale-Biscoe 1979), early research showed that the blastocyst does not extend CL lifespan (Hinds and Tyndale-Biscoe 1982). Likewise, the differences in progesterone levels in pregnant versus non-pregnant cycles are caused directly by a placental signal, as it has been shown for the koala (Johnston et al. 2000) or in the quokka (Cake et al. 1980) and tammar (Hinds and Tyndale-Biscoe 1982) early gestation. With exceptions, female marsupials have pregnant cycles shorter than their oestrus cycle (Tyndale and Renfree 1974, Merchant 1979). This observation led to the hypothesis that early progesterone falls at the end of the pregnant cycle could result from the luteolytic effect or a luteolytic signal from the fetus (Hinds and Tyndale-Biscoe 1982b). In this regard, Hinds proposed a model according to which the release of endometrial prostaglandin triggered by fetal glucocorticoids causes pulses of pituitary prolactin (Hinds 1990). The latter would be responsible for shortening CL lifespan only during pregnancy. Based on this evidence, Renfree (2000, 2010) considers that while *endometrial* recognition of pregnancy is ancestral in marsupials (present in basal branching *Monodelphis domestica*), *maternal* recognition of pregnancy, defined in this context as as any type of influence through systemic signals of the fetal-placental unit on CL lifespan, is likely a derived trait in Macropodidae. While these results suggest that the embryo of at least some marsupial species has the ability to signal its presence and influence ovarian function, the signal, as opposed to the ones involved in eutherian MRP (reviewed in Bazer 2015), reduces CL lifespan (Bradshaw and Bradshaw 2001).

If we follow Short's classical definition of MRP as a mechanism that prolongs the luteal progesterone production, we find that the phenomena is not ubiquitous even among eutherians, and only if understood more broadly (as endometrial recognition) can it be considered a universal eutherian feature. In fact, some eutherians do not prolong the CL function in pregnancy - these are primarily species with long non-pregnant luteal phases to encompass pregnancy (e.g., dog), thus lacking typical mechanisms for maternal recognition of pregnancy. These cases could resemble viviparity in non-mammalian vertebrates or gestation in marsupials, and result from gestation lengths short enough to be encompassed by the ancestral luteal phase in the lineage. Alternatively, such long non-pregnant luteal phases might have evolved secondarily, if the extended pCL lifespan resulted in a prolonged npCL too, particularly in cases in which selective pressures to shorten the npCL may be missing, such as in seasonal reproduction or synchronized breeding in the group.

Three patterns of npCL lifespan can be distinguished: long-lived, short-lived, and ultra-short lived (Hennebold 2018; **Figure 2**, adapted). As said, species with long-lived CL show no substantial differences between the pregnant and non-pregnant states, a situation also known as *physiological* or *covert* pseudopregnancy. In the short-lived corpora lutea, the CL lifespan is prolonged in response to conceptus, compared to the non-pregnant cycle. This type is found conspicuously in most eutherian species and every superorder. On the other side of the spectrum are many rodents, in which the non-pregnant luteal phase is very short and likely secondarily reduced for fast reproduction. In species with an ultra-short luteal phase, a fully functional npCL does not fully develop, and a functional luteal phase can only be induced, mostly by mating. In the case of sterile mating a *true* pseudopregnancy develops, meaning a pregnancy-like luteal phase that can be induced but is not a part of the regularly occurring cycle. Finally, in species with reflex ovulation, ovulation is also induced by mating, and hence, the CL of pregnancy or nonpregnancy (if unfertilized), only develops after mating. Induced ovulation does not show specificity for any order, as it is found in Lagomorpha, Carnivora, Artiodactyla, Perissodactyla, and Eulipotyphla, and might be ancestral (Pavlicev and Wagner 2016). Here, we review the types of ovarian cycles based on luteal CL lifespan across eutherians, as well as important information on their physiology of reproduction.

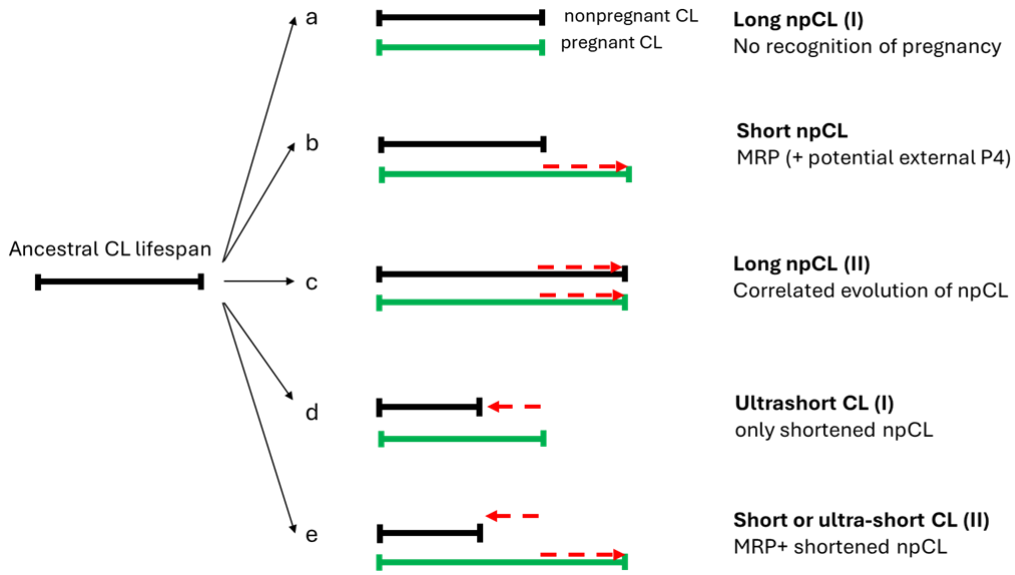

Figure 2. Scheme with the plausible modifications of the lifespan of the corpus luteum of pregnancy (pCL) relative to the nonpregnant CL (npCL). MRP: maternal recognition of pregnancy.

4.3. Review of the luteal phase and maternal recognition of pregnancy in placental mammals

Xenarthra

Although most xenarthran species prolong the lifespan of the CL in pregnancy, to our knowledge, the mechanisms for maternal recognition of pregnancy in armadillos, sloths and anteaters, are unknown.

The xenarthtran order Cingulata include the nine-banded armadillo (*Dasypus novemcinctus*), with an invasive, villous hemochorial placentation (Enders and Carter 2012), a delayed implantation and polyembryony (Hamlett 1933, 1935). The female has a single annual ovulation, and fertilization is followed by an embryonal diapause, delaying implantation by 3 to 5 months (Talmage and Buchanan 1954). Progesterone levels during embryonic diapause do not differ from those of non-pregnant animals (Labhsetwar and Enders 1968), but removing both ovaries during the diapause period precipitates implantation via unknown underlying mechanisms (Buchanan 1956). The pCL shows signs of regression in the latter part of gestation, and progesterone is supplemented by the fetal adrenal gland, whereas the trophoblast is only weakly steroidogenic (Nakakura et al. 1982). Authors have suggested that armadillo's reproductive features hint that the ancestral ovarian cycle entailed a long, intrinsically regulated luteal phase, into which the invasive implantation and placentation in eutherians have evolved (Hennebold 2018). However, studies of the ovarian cycle of different armadillo species have reported short-lived, functional corpora lutea. For example, authors have indicated that the oestrus cycle of the six-banded armadillo (*Euphractus sexcinctus*) is around 26 days (Campos et al. 2016) and about 25 for the three-banded armadillo (*Tolypeutes matacus*). The dissociation of these traits could explain the observed plasticity in type of ovulation (induced vs. spontaneous) and number of yearly cycles (monoestrous vs. polyestrous) in response to environmental cues, without implying a difference in the presence/absence of maternal recognition of pregnancy (MRP) (Stephens et al. 2013).

Representatives of the other xenarthran order, Pilosa (anteaters and sloths), also show a cyclicity and a short-lived, functional npCL. For example, Giant anteaters (*Myrmecophaga tridactyla*) are polyestrous, nonseasonal breeders with villous hemochorial placentas and the gestation period of around 6 months. Their regular ovarian cycles are around 53 days long, with the luteal phase of about 2-3 weeks and regular preovulatory estrogen peaks followed by increasing progesterone levels (Knott et al. 2013, Patzl 1998). Similarly, the two-toed sloth (*Choloepus didactylus*) has regular nonseasonal cycles of around 30 days, with short-lived corpus luteum (luteal phase of 11-16d) and pregnancy of 10 months (Troll 2013, Taube 2008). Shorter cycles of around 16 days have been suggested for the Hoffmann's two-toed Sloth (*Choloepus hoffmanni*; Calvo-Fernandez et al. 2024), with the same gestation length. Gestation length of the sloth genus *Bradypus* with more emphasized seasonality, the gestation length is around 6 months (Taube et al. 2008). Sloths also possess labyrinthine endotheliochorial placentas (Carter and Moss 2012). Although most xenarthran species must possess ways of prolonging the lifespan of the CL in pregnancy, to our knowledge, the underlying mechanisms are unknown. Information on this basally branching clade would be particularly important, in order to reconstruct the evolution of maternal recognition of pregnancy in terms of either prolonged lifespan of corpus luteum, or the co-option of different steroidogenic tissues.

Afrotheria

Within Afrotheria, the species within the clade of Elephants, Hyraxes and Sirenians (Paenungulata) show similar reproductive traits. Elephants (order Proboscidea) have particularly long sterile ovarian cycles, as well as gestational lengths. African and Asian elephants prolong their pregnant progesterone levels by accumulating up to 42 CL in the ovary despite uniparity (Perry 1953; Smith et al. 1969), as they produce accessory CLs (acCLs) from non-ovulated, luteinized follicles (Laws

1970). The accessory corpora lutea develop are essential for maintaining the long elephant pregnancies of around 22 months, during which the ovary is the only source of progesterone (Lueders et al. 2012). In the absence of an embryo, the elephant CL begins to regress after six weeks, constituting a conspicuously long luteal phase. The rescue of Elephants CL occurs once a viable blastocyst attaches to the endometrium, rather than during the preimplantation phase, and is thus characterized as a last-minute mechanism for pregnancy recognition (Lueders et al. 2012). A luteotropic placental lactogen is the main candidate for the mechanism for maternal recognition of pregnancy in the African elephant (Yakamoto et al. 2011).

Species of the closely related order Sirenia (dugong, manatee) show similar reproductive patterns: accessory corpora lutea, uniparity, relatively long ovarian cycle (46-64 days in dugong; Wakai et al. 2002), long gestational length of 12-13 months, and endotheliochorial placentation (Marsh 1984, Rodrigues et al. 2008, Carter et al. 2008). In contrast, the Rock hyrax (*Procavia capensis*, order Hyracoidea*)* lacks accessory CL; instead, the CL forms only from ovulated follicles. Based on vaginal smear composition, their cycles are estimated to be of around 14 days (Gombe 1983). Hyracoidea species show remarkably long pregnancies (around seven months) for their small size and have hemochorial placentation (Kayanja 1973). It is unclear whether sources other than the CL provide progesterone for the full length of pregnancy. However, early reports indicated the presence of chorionic gonadotropin-like hormone in the hyrax placenta (Charanjit 1980).

Less is known about the oestrus cycle in the afrotherian order of afrosoricids, consisting of tenrecs, golden moles and otter shrews. Tenrecs breed seasonally (Gould and Eisenberg 1966; Nicoll and Racey 1985) and have long gestations (50-70 days). For example, small madagascar hedgehog tenrec *Echinops telfairi* is seasonally polyestrous, with short recurring oestrus cycles of six days (Godfrey and Oliver 1978) and long gestations of 62-65 days (Eisenberg and Muckenhirn 1968). Details of potential pseudopregnancy or the extraovarian progesterone sources are unknown.

Lastly, the afrotherian order Macroscelidea includes a well-studied species, the eastern rock elephant-shrew (*Elephantulus myurus*). Interest in this species is due to its spontaneous decidualization, menstruation, and invasive placentation (Carter 2018). The estrous cycle of the elephant shrew is about 12 days, with a short functional luteal phase that is extended in the pregnant cycle to a gestation length of 24 - 75 days (51 in *E. myurus*; Tripp 1972). Aardvark (Tubulidentata), has a long gestation (7 months) and seasonal reproduction (Van Aarde 1984). To our knowledge, the mechanisms for MRP or placental steroidogenesis have not been studied in elephant shrews or aardvark.

No reported cases of luteal-placental shift in Afrotherian species appear to have been encountered thus far. Overall, the most common pattern in the Afrotherian orders is that of a short, functional npCL and a long gestation.

Laurasiatheria: ungulates, bats and insectivores

In this group, economically valuable ruminants are particularly well studied. Ruminants have a short-lived corpus luteum that is extended in pregnancy. In cattle and sheep, interferon tau (IFNT) has been identified as the primary signal for the extension of CL in pregnancy (MRP). This cytokine, produced by the embryonal trophectoderm (TE) cells in the peri-implantation period, rescues the CL via multiple mechanisms (Roberts et al. 1992). The best-known mechanism is by silencing the estrogen receptor alpha (ESR1), and thereby downregulating oxytocin receptors in the endometrial epithelium thereby disrupting oxytocin-induced release of endometrial prostaglandin PGF2α (Bazer et al. 2008). Less explored mechanisms by which IFNT extends CL lifespan include inhibiting lymphocyte proliferation, modifying the PGE2:PGF2α ratio by prioritizing PGE2 biosynthesis, or altering prostaglandin transport to the CL (Arosh et al., 2004, 2016). In addition to maintaining CL, the bovine placenta also contributes to progesterone production towards late pregnancy (Shenavai et

al., 2010). In sheep, the expression of endogenous Jaagsiekte sheep retroviruses (EnJSRVs) in the endometrial luminal and glandular epithelium is enhanced by luteal progesterone and stimulates trophoblast proliferation and production of IFNT (Spencer et al. 2006, Spencer and Bazer 2002). In addition to luteal progesterone, the ewe presents a luteal-placental shift at around 55 days of gestation (Casida and Warwick 1945).

The pig ovarian cycle lasts 18-21 days, with the CL forming 4-5 days post ovulation, and peaking in progesterone production at day 12-14 in nonpregnancy. Removal of the uterus in the mid-luteal non-pregnant phase results in a CL lifespan of 114 days or more (Anderson 1973), implicating uterine production of a luteolytic factor, likely PGF2α. Bazer and Thatcher (1977) proposed that the endocrine secretion of endometrial PGF2α of the non-pregnant cycle becomes exocrine in pig pregnancy, secreted into the uterine lumen thereby interrupting its luteolytic potential (the *endocrine-exocrine theory* of MRP). The substance secreted by the pig conceptus that causes this change is estradiol-17 β (E2) (Bazer et al. 2008, 2013). In addition, the pig conceptus also increases the PGE2/PGF2α ratio by increasing PGE2 levels, and releases cytokines and interferon-γ (IFNG) and IFN-δ (IFND) into the uterine lumen at the time of implantation (Ka et al. 2018). However, these have no anti-luteolytic effect but rather prime the endometrium for implantation. Finally, studies have reported increasing levels of placental progesterone production throughout gestation (Knight 1994), suggesting a progesterone complementation by the fetal tissue.

The camelid family possesses several unique features among even-toed ungulates (artiodactyls), such as induced ovulation followed by the development of a short-lived CL. In the induced nonpregnant cycle (i.e., pseudopregnancy), progesterone peaks ten days after ovulation, with endometrial luteolytic pulses of PGF2α starting to peak a day later. If fertilized, rescue of CL of pregnancy in the dromedary (with an ovarian cycle of about 28 days; Musa 1979) occurs between days 8 and 12 after mating, and the CL is the only source of progesterone throughout 15-month-gestation (Skidmore 2018). Although the exact mechanism of CL extension is not established, it has been suggested to also involve blastocyst estrogen release and endometrial estrogen receptors (Bianchi 2022).

Slightly longer ovarian cycles have been reported for aquatic mammals, the Cetaceans. All the studied Cetaceans are spontaneous ovulators with a short, functional npCL and extended CL of pregnancy, however the mechanism of this extension is unknown (Fukui 2007, Pomeroy 2011, Robeck and O'Brien 2017). For example, the nonpregnant cycles of the atlantic bottlenose dolphin (*Tursiops truncatus*) and pacific white-sided dolphin (*Lagenorhynchus obliquiden*) are of around 27 days, respectively 31 days (Robeck et al. 2005; Robeck et al. 2009), bit gestational lengths in these species are around 12 months. Even longer sterile cycles of about 40 and 50 days have been described in orca (Robeck and O'Brien 2017) and beluga whale (Steinman et al. 2012), which also have longer gestations (17, respectively 14 months). The steroidogenic capacity of the cetacean placenta has been shown in single species, such as in the Minke whale (*Balaenoptera bonaerensis*, Sasaki et al. 2013) or Killer whale (*Orcinus orca*, Robeck et al. 2018). In short, the ovarian cycles of the even-toed ungulates including aquatic mammals entail a relatively short, functional corpus luteum in nonpregnancy, with oestrus cycles ranging from 15 (e.g., lesser mouse deer *Tragulus javanicus,* Kusuda et al. 2013) to around 50 days in some cetaceans, which is substantially prolonged in pregnancy and often complemented by the placental progesterone.

Odd-toed ungulates (perissodactyls: horses, rhinoceros, tapirs) manifest seasonal spontaneous polyestrous nonpregnant cycles of 21-22 days and a progesterone peak around 6-18 days post-estrus. The mare conceptus releases an independently evolved chorionic gonadotropin, equine chorionic gonadotropin (eCG) (Carter 2022) that binds to LH receptors. eCG appears only around day 35 in pregnancy, prolonging the pregnancy by inducing additional ovulations and promoting the formation of accessory corpora lutea (Murphy 2018), being a late mechanism of pregnancy recognition, likely

evolving in addition to an earlier mechanism. In the mare, the conceptus inhibits the endometrial release of PGF2α by an unknown underlying mechanism (Klein and Troedsson 2011), for which several possibilities have been suggested (Lawson 2022, Smits 2020). By week 16, the mare presents a luteal-placental shift (Conley 2016). The sumatran rhinoceros (*Dicerorhinus sumatrensis*), on the other hand, is the only reported representative of this clade to have induced ovulation (Roth et al. 2001). Rhinos possess a short-lived functional CL, but exact length of the ovarian cycles can vary widely across species, ranging from 21 days in Asian rhinos, 43 days in Indian rhinos or up to 70 days in white rhinos (Roth and Brown 1999, Schwarzenberg et al. 1998, 2000). Lastly, a luteal-placental shift after the third month of gestation has been reported for several rhinoceros' species, with placental progesterone until the end of 16-17-month pregnancy (Schwarzenberger and Hermes 2023).

The speciose order of bats (Chiroptera) manifests broad variation in ovarian cycles: in temperate zones, many species are monoestrous (e.g., the Schlieffen's bat, Van der Merwe and Rautenbach 1987; Common bent-wing bat, Bernard et al. 1991), whereas polyestrous cycles are more common among the tropical species (e.g., wild fulvous fruit bat, Zhang et al. 2007; Cave nectar bat, Krutzsch 2005). Several families include species with spontaneous decidualization in nonpregnancy and menstrual-like bleeding, among them the basal branching Pteropodidae (e.g., the short-tailed fruit bat *Carollia perspicillata*; Rassweiler and Bonilla 1992), Molossidae (Rassweiler 1991), and Phyllostomidae (Hamlett 1934). These species have ovarian cycle lengths of around thirty days (Rasweiler and Bonilla 1992, Zhang et al. 2007). Bats can modify their reproductive cycle through delayed ovulation, implantation or embryonal development, and during the latter two the activity of CL is reported to be reduced (Crichton et al. 1989), which complicates determination of the length of pregnancy and pregnant CL. Progesterone concentration peaks close to parturition in most species examined, such as in the big brown bat (*Eptesicus fuscus*, Greville et al. 2022), or in the little brown bat (*Myotis lucifugus*, Currie et. al. 1988). Placental progesterone synthesis is known in several species, such as in the Natal clinging bat (*Micropterus schreibersii natalensis*, Van Aarde et al. 1994) and in the little brown bat (Currie et. al. 1988). Signs of structural luteolysis are reported to significantly precede parturition in bat species, which is in itself not surprising given placental progesterone synthesis. Intriguingly, in some species, systemic progesterone levels are reported to start decreasing in mid pregnancy (Burns and Easley 1977), but this may be related to a developmental delay. Overall, bats manifest a short npCL, which is prolonged in pregnancy, and additional placental progesterone. Bats also presents an extensive range of invasiveness, from endotheliochorial to invasive hemochorial placentation (Gopalakrishna and Karim 1979).

Among carnivores, the families of the suborder Caniformia differ substantially in the length of npCL, yet all species studied present endotheliochorial placentas (Enders and Carter 2012). The reproductive biology of the Mustelidae family is known for its widespread obligate embryonic diapause. Ferret (*Mustela putorius*), for example, is seasonally polyestrous with copulation-induced ovulation leading to pregnancy or pseudopregnancy of 39-42 days (Deanesly 1967), and the luteal progesterone levels that start to decline midpregnancy, much before structural luteolysis occurs. The European mink (*Mustela lutreola*) is seasonally polyestrous (Amstislavsky et al. 2009 a,b) and has a breeding season with oestrus lasting from 1 to 12 days and, when not mated, can re-enter new cycles after 2 to 4 weeks. As is in other Carnivorans, there is no evidence of steroidogenesis in the mink or ferret placentae (Douglas et al. 1998, Blatchley and Donovan 1976). Most species of the Ursuidea family are seasonally polyestrous, induced ovulators (Spady et al. 2007, Boone 2004). Most bears have embryonic diapause, with the CL and the embryo entering a dormant phase after fertilization, enabling the female to enter a new cycle and mate repeatedly, potentially accumulating a litter with different fathers. The litter implants simultaneously after the diapause is finished. The long CL has been reported in seasonally spontaneously ovulating black bears (Tsubota et al. 1987) if no matings occurred, similar to Canids. No placental progesterone synthesis has been found in bears (tested in

Japanese black bear, Sato et al. 2001), and MRP is unknown. Seasonal monoestrous cycles with a long npCL are common also in seals (Pinnipedia superfamily; Boyd et al. 1991), with exemptions (e.g., the polyestrous Hawaiian monk seal *Monachus schauinslandi*; Iwasa and Atkinson 1997). No placental progesterone synthesis was found in the seal species examined (Ishinazaka et al. 2001). Like ursids and mustelids, most species of Pinnipedia have embryonic diapause (Renfree and Calaby 1981).

The dog-like carnivores (canids) present several unique characteristics, such as obligatory periods of ovarian inactivity or anestrus (Nagashima and Songsasen 2021). Most species are spontaneous ovulators, with some exceptions (e.g., the maned wolf, *Chrysocyon brachyurus*; Johnson et al. 2014). Canids have a long monoestrous cycle, with a luteal phase of around two months, irrespective of pregnancy (Concannon 2018). Although the uterus does not shorten or lengthen the sterile ovarian cycle, as shown by its removal, prostaglandin PGF2α does cause a prepartum luteolysis in pregnancy, frequently leading to the pregnancy being somewhat shorter than the sterile luteal phase (Kowalewski 2014). In contrast, in cat-like carnivores (felids), the pregnant and nonpregnant luteal phases diverge, displaying differences in progesterone patterns. This suggests a role for the luteotrophic and luteolytic factors such as relaxin, prolactin, and prostaglandin PGF2α (Banks et al. 1983, Tsutsui et al. 1993, Siemieniuch et al. 2010). Felids are mostly induced ovulators but also include species such as lynx, reported to ovulate spontaneously under natural conditions (Brown 2006; Painer et al. 2014). Many felids display polyestrous cycles (Wildt et al. 1981). The domestic cat (*Felis catus)* has induced ovulation and can enter a new oestrus cycle after 2 to 3 weeks if the mating did not lead to fertilization. After a fertile mating, the cycle is prolonged, with pregnancy lasting 58 - 65 days. Other felids have slightly longer cycles and gestation periods, like the Bengal tiger (*Panthera tigris*) with over 100 days gestation (Brown 2011). The Eurasian and Iberian Lynx are interesting exceptions, with a long monoestrous cycle and a CL lifespan of two years or more, with elevated progesterone levels (Painer et al. 2014; Göritz et al. 2009). Oestrus and pregnant cycles (44, respectively about 50 days) are close in length also in the bobcat (*Lynx rufus*; Göritz et al. 2009). Studies have reported the presence of steroidogenic enzymes and detectable levels of progesterone in the felid placentae for domestic cat and Iberian lynx (Malassiné et al. 1979, Braun et al. 2012), but the details of the placental progesterone complementation are not well-known.

Finally, within the order of insectivores (Eulipotyphla), little direct information on the lifespan of corpus luteum could be found. We encounter among insectivores a very short estrous cycle (2-4 days) in the seasonally polyestrous short-tailed shrew (*Blarina brevicauda*; Nowak 1999), with gestation average of 21 days. On the other hand, the Hispaniolan solenodon (*Solenodon paradoxus)* has a 9-13 day estrous cycle, which is extended in gestation to, on average, 50 days (Eisenberg and Gould 1966).

In summary, the reproductive cycle of Laurasiatherians encompasses a great variation of regimes, including the ultra-short, relatively short and a long-lived npCL. In some of the species with short-lived npCL, above all the ungulates, anti-luteolytic mechanisms for maternal recognition of pregnancy have been identified, but in most orders they remain unknown.

Primates and rodents: Euarchontoglires

Euarchontoglires is the mammalian clade including the primates and their relatives (Euarchonta), as well as rodents and lagomorphs (Glires). Reproduction of the rabbit (*Oryctolagus cuniculus)* is best investigated among lagomorphs. Rabbits undergo mating-induced ovulation with the subsequent formation of a CL of pregnancy (30 days) or pseudopregnancy (16-18 days; Bazer 2015). The CL persists as the primary source of progesterone throughout pregnancy. When and how the function of the CL in pregnancy is rescued beyond the lifespan in pseudopregnancy is not known (Browning and Wolf 1981, Marcinkiewicz et al. 1992, Gadsby et al. 1983), as no significant differences between

pregnant and pseudopregnant females until days after implantation have been found. Estrogen has been suggested as the main luteotrophic factor (Keyes and Gadsby 1987).

Rodents are the largest mammalian group, with a highly diverse reproductive biology. In the following, we use the phylogeny of Swanson et al. (2019), staying at the suborder level. The basally branching suborder of Ctenohystrica (Guinea pig, chinchilla, viscacha, degu, porcupine, etc.) is characterized by spontaneous ovulation, a functional nonpregnant CL, and a long gestation. For example, the Guinea pig (*Cavia porcellus*) has a 16-day cycle, 12 of which are the luteal phase, and a gestation length of 70 days. A release of a luteotropic substance three days after ovulation induces the formation of functional CL, while the second release depends on embryo implantation between days 4 and 6 of gestation, maintaining the CL until mid-gestation, when luteal-placental shift occurs and placenta provides the progesterone until parturition (Aldred et al. 1961). The maintenance of the CL beyond the luteal phase of the nonpregnancy has been suggested to be related to the presence of a chorionic gonadotropin-like hormone (Sherman et al. 2001, Carter 2022) or a prolactin-like hormone secreted by the spongiotrophoblast (Alam et al. 2010); however, the exact mechanism remains unknown. Chinchilla has a long non-pregnant cycle of 30-40 days, and 105-115 days gestation, similar to plains viscacha (*Lagostomus maximus*), with a 45-day estrous cycle and a 154-day gestation (Weir 1971). In degu (*Octodon degus*), the cycle is 17-21 days, while gestation lasts 90-95 days (Reynolds and Wright 1979). Naked mole rats (*Heterocephalus glaber*) have a gestation length of 70 days.

Similar gestation lengths can be found in the suborder of squirrel-like rodents (Sciuromorpha). In the Arena Mountain Beaver (*Aplodontia rufa nigra*) gestation lengths of up to a month have been reported, although they may be underestimated (for a discussion see Zielinski and Mazurek 2016). Similar is the case with the monoestrous Alpine marmot (*Marmota marmota*: 33-35 days) or Woodchuck (*Marmota monax: 34 days*), as well as ground squirrels (28-34 days), and somewhat longer in squirrels (up to 42 days). The length of the estrous cycle or specifically of the luteal phase is not reported in most members of Sciuromorpha. In the suborder Castorimorpha, the nonpregnant cycle suggests short, functional npCL, and these species also manifest a long pregnancy: Beaver (*Castor sp.*), a seasonally polyestrous species with a cycle of around two weeks, and a pregnancy of 100-130 days or the Kangaroo rat (*Dipodomys* sp.), with a cycle of 12-13d and a gestation length of ca. 31days.

Notably, many rodents with relatively long nonpregnant cycles breed seasonally in the wild- possibly indicating that their npCL is under less selection for rapid succession of estrous phases- even if they may be physiologically able to breed continuously in captivity.

Classical rodent model species belong to the superfamily Muroidea in the suborder of mouse-like rodents (Myomorpha): rats, hamsters, deer mice, gerbils and mice. These species are characterized by a short (4-5d) nonpregnant cycle with an ultra-short, nonfunctional npCL. They lack chorionic gonadotrophins and placental progesterone production. In the paradigmatic animal model, the house mouse (*Mus musculus*), the progesterone from the nonpregnant postovulatory follicle is low, and the follicle soon degenerates. Mating is necessary in house mouse for a functional CL with sufficient progesterone for implantation to develop. Mating induces the release of prolactin (PRL) from the anterior pituitary, the initial luteotrophic signal enhancing LH receptors and inhibiting the activity of progesterone-catabolizing enzyme (20α-hydroxysteroid dehydrogenase; Soares 2004). If mating is sterile, this results in 12-14 days long pseudopregnancy. If mating is fertile, hormones from the conceptus and the uterine decidua maintain the luteal cells and progesterone secretion after day 12, shifting the dependency of pregnancy to placental luteotrophins (Choundary and Greenwald 1969). While its function in lactation is shared among eutherians, prolactin (PRL) has a broad species-specific array of additional functions (Soares at el. 2007), and the involvement in pregnancy of PRL gene family members have been reported in rodents, primates, and ruminants (Soares 2004),

even if their exact roles are not fully understood (Ben-Jonathan et al. 2008). In most species, PRL production is restricted to the pituitary and the decidua; however, in mice, trophoblasts also express PRL genes (Rawn and Cross 2008). Pituitary PRL plays a similar, if not identical, role as the pregnant luteotrophic hormone in hamsters and rats. Cricetidae hamsters and rats also have ultra-short npCL, with short estrous cycles of four days and gestation periods of 16 and 22 days, respectively. They have induced pulses of prolactin from the maternal anterior pituitary gland and lactogenic hormones from the uterine decidua and placenta. While shared among (most efficiently reproducing) model species, this type of cycle is not representative of all Myomorpha. Indeed, even within the Muridae family, several species show long nonpregnant cycles with functional luteal phase, such as the Spinifex hopping mouse (*Notomys alexis*) with a 8 days cycle (Breed 1975, Breed et al. 2011), or the African spiny mice (*Acomys cahirinus*), with 6 - 10 days, and a menstruation of ca. three days at the end of the luteal phase (Peitz 1981, Bellofiore et al. 2018). A functional npCL also exists among Critecidae, e.g., the Cotton rat (*Sigmodon hispidus*) has a 7–9-day cycle (Faith et al. 1997). Interestingly, these species also have a longer gestation than the species with ultra-short CL (38-41 days in N. *alexis*, 38-45 days in *A. cahirinus, 27*-28 days in *S. hispidus*). The presence of a luteal-placental shift or mechanisms of MRP have not been reported for these species.

Primates are characterized by a short-lived functional npCL which is extended during pregnancy. The maternal recognition of pregnancy is well understood in primates. The syncytiotrophoblast of the blastocyst adhering to the maternal endometrium secretes chorionic gonadotropin (CG) in New World monkeys, Old World monkeys, apes, and humans. This hormone is closely related to pituitary luteinizing hormone (LH), part of the maternal hypothalamic-pituitary-ovarian axis of all mammals, sharing with it structural alpha-subunit, and binding to the same receptor (LHCGR). The gene for its β-subunit arose by the duplication of the β-subunit gene of the luteinizing hormone in the lineage of anthropoid primates (Maston and Ruvolo 2002). In humans, CG (hCG) is transcribed at the eight-cell stage before implantation and the hCG signal replaces the luteotrophic effect of LH, signaling via the same receptor and maintaining the corpus luteum beyond the lifespan of nonpregnant state (Sugino et al. 2000, additional factors could be involved in promoting human CL's steroidogenesis after implantation (Oon and Johnson 2000). Eventually the primate corpus luteum nevertheless degrades (after approximately 8 weeks, rather than 10 days for npCL in humans). Subsequently, progesterone level is upheld by the shift to fetal placental progesterone production (i.e., luteo-placental shift) at about seven weeks of human pregnancy (Fazleabas et al. 2004).

Although with some specializations (which may or may not have a parallel in Old World primate adaptations), such as more variation in litter size, New World monkeys exhibit typical primate cycles. For instance, the Marmoset (*Callitrhix jacchus*) has cycles of around 28-30 days (but lacks menstruation) and gestation lengths of around 144 days (Tardif et al. 2003). It was reported early on that the duration of CL's progesterone secretion is longer in the Marmoset than other primates, indicating that the luteal-placental shift occurs at a later stage of pregnancy (Hodges et al. 1983). (Hodges et al. 1983). Interestingly, it has been proposed that a higher number of fetuses (twins in nature) may extend the period of elevated chorionic gonadotropin, resulting in a delayed luteal-placental shift (Ziegler et al. 2023).

Prosimian primates (lemurs and their relatives) do not produce chorionic gonadotropin. In addition, strepsirrhine primates possess epitheliochorial placentas and non-invasive implantations (Mossman 1987, Carter and Enders 2003). Prosimians have nonseasonal spontaneous ovarian cycles, as well as long gestations, like other primates do (reviewed in Van Horn and Eaton 1979). For instance, the mean ovarian cycle length of the ring-tailed lemur (*Lemur catta*) is around 39 days, with gestations of about 136 days (Evans and Goy 1968). On the shorter end, the Philippines tarsier (*Carlito syrichta*) has cycles of about 23 days and gestations of about six months (Catchpole and Fulton 1943). Some prosimians, such as the brown greater galago (*Galago crassicaudatus*), have

conditional seasonality: this nocturnal primate is polyestrous in captive conditions while remaining monoestrous in the wild (Von Horn and Eaton 1979). Their oestrus cycle length, however, is within the range for primates (about 44 days) (Eaton 1973), with a gestation of 133 days, illustrating that short-lived npCL can coincide with monoestrous reproductive modes. To our knowledge, nothing is known about the prosimian luteo-placental shift, nor about the mechanisms that extend CL lifespan in prosimians.

In summary, most Euarchontoglires manifest a short-lived functional npCL. A subset of rodents, including several animal models, have a likely derived ultra-short npCL with an induced luteal phase. No cases of long-lived corpora lutea have been reported in this superorder. Prolactin and chorionic gonadotropin are the most widespread signals for maternal recognition of pregnancy in this superorder, but they are also the only ones studied to date. The signals involved in maternal recognition of pregnancy in rodents with a short functional npCL, or in prosimians, are unknown.

5. Evolutionary trends in mammalian CL lifespan and life history traits

The endocrine participation of the fetus, first through signals to prolong the corpus luteum and then through progesterone synthesis, introduced a shift in the control of pregnancy support from the ovary to the blastocyst and then to the placenta. These two mechanisms are two steps of a crucial event, namely the decoupling of the pregnant and nonpregnant cycle, and constitute essential novelties in neuroendocrine control that paved the way for the longer gestation lengths, characteristic of placental mammals. Still, understanding the evolution of pregnancy and its decoupling from the serially homologous luteal phase of nonpregnancy requires considering what may have driven the evolution of eutherian gestation length before and after the appearance of these signaling innovations. Several observations can be made based on the presented review.

First, the comparison of phylogenetic distributions of pregnant and nonpregnant cycle length with other reproductive traits shows that the maternal recognition of pregnancy is widely distributed, co-occurring with any degree of invasiveness of placentation and even induction of ovulation. The mechanisms of decoupling, such as endocrine activity of the blastocyst and the placental progesterone production are present in species with invasive placentation (e.g., primates, rodents), as well as in the lineages with noninvasive placentation (e.g., ungulates; **Figure 3)**. A short-lived, functional npCL is the most widely distributed type of cycle among extant eutherians, similarly co-occurring with all degrees of invasiveness of placentation, the presence of luteal-placental shift, pseudopregnancy and the type of ovulation **(Figure 3)**. The long-lived npCL in long monoestrous cycle, is the rarest of all cycle types and coincides with an endotheliochorial placentation in carnivores. The significance of this coincidence is questionable, due to the rare occurrence of the long cycle. Similarly lineage-specific is the ultra-short-lived npCL and induced-luteal phase (Muridae, Cricetidae, Eulipotyphla, Afrosoricida and Macroscelidea), which only seem to have evolved in species with hemochorial placenta with no progesterone expression **(Figure 3)**. From the nestedness of the species with exceptionally long or ultra-short npCL in clades with short-lived npCL, we suggest that the former two are derived cycle types, whereas the latter is the shared condition for most major groups, following the origin of the prolonged corpus luteum lifespan in pregnancy (MRP).

Second, different mechanisms of maternal recognition of pregnancy are encountered in major lineages **(Figure 4)**, which strongly suggests that these have originated independently to decouple the pregnant from nonpregnant cycle, after the lineages separated.

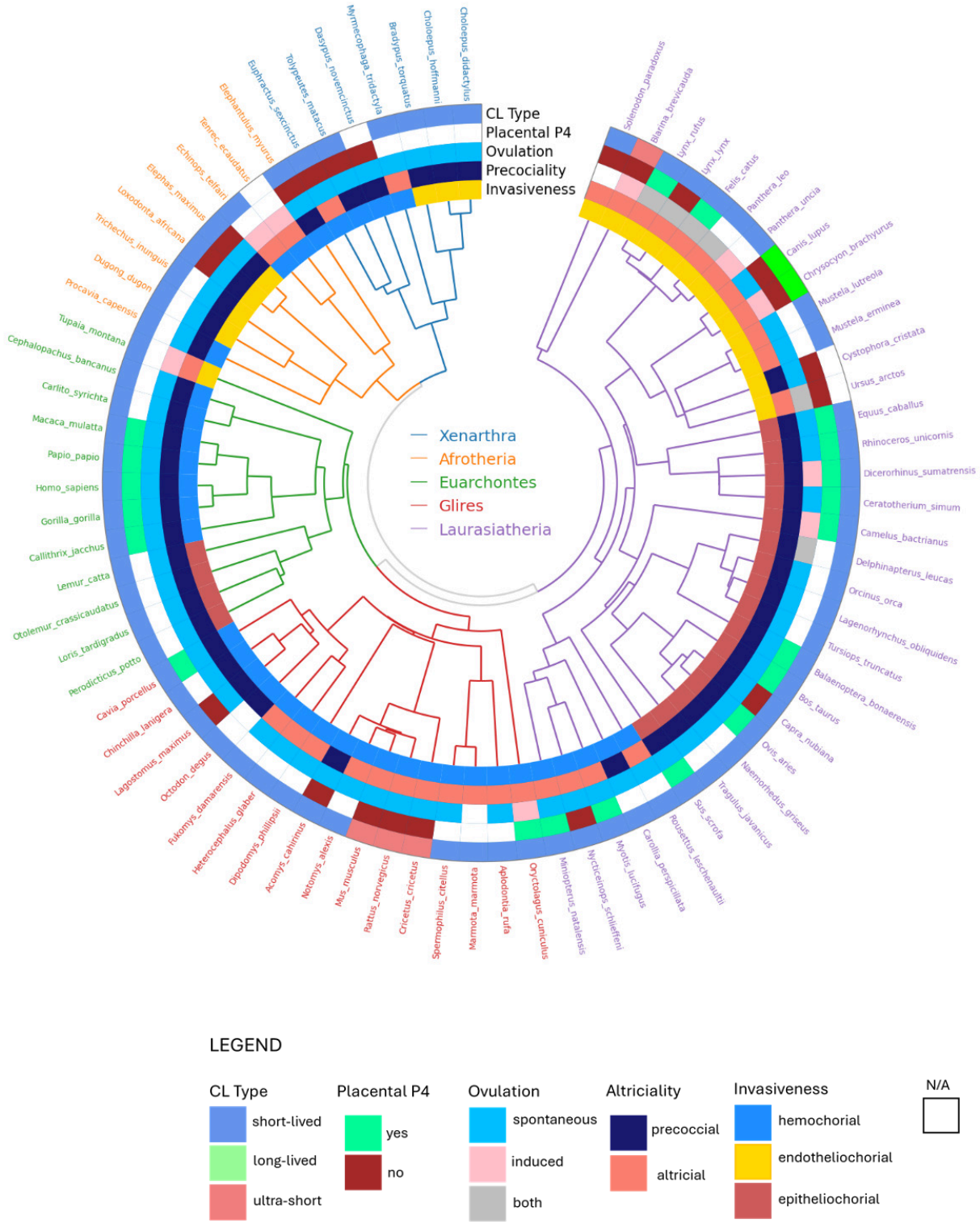

Figure 3. Character state matrix for five reproductive characters (CL type, placental progesterone, ovulation type, altriciality and degreee of invasiveness: see data in Supplementary Data 1) plotted along the phylogenetic tree of placental mammals (timetree: Kumar et al. 2022). Branches are coloured according to superorders (center key).

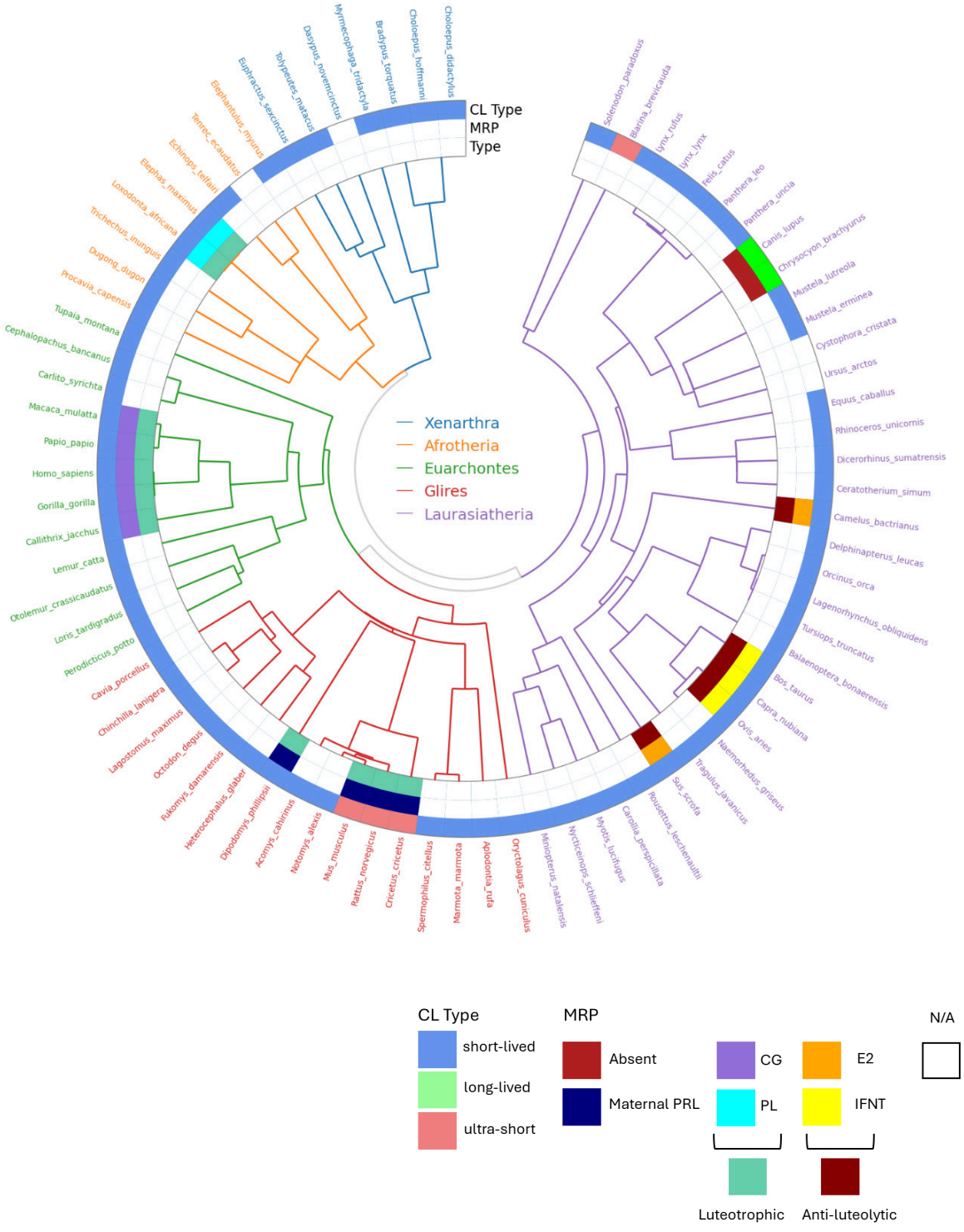

Figure 4. **No single control mechanism can be used to explain corpus luteum lifespan across eutherians**. Character state matrix for the CL type along with mechanisms for MRP plotted along the phylogenetic tree of placental mammals (see Supplementary Data 2). Although unknown for most species, there is no universal factor that rescues the CL. Signals can have local or systemics effects and be luteolytic or anti-luteolytic. Note that MRP is absent in those species long enough to encompass pregnancy and that in species with very short cycles it may be governed by maternal hypophysial prolactin (PRL). CG: chorionic-gonadotropin; PL: placental lactogen; E2: estrogen; IFNT: interferon-tau.

Third, it has long been appreciated that eutherian evolution involves increases in body size (Cope 1887, Alroy 1998) and that the gestation length scales allometrically with body size in species giving birth to precocial as well as altricial young, albeit to a different extent (Martin and MacLarnon 1985). These relationships are also found in our dataset, despite its smaller size, owing to the condition that pregnant as well as nonpregnant cycle lengths are known **(Figure 5A)**. Moreover, we found that the length of the nonpregnant cycle is also positively correlated with body size **(Figure 5B)**. The latter implies that the length of the nonpregnant cycle coevolved with the gestation length as their correlation is reduced, but maintained even when accounting for body size (by regression on log-log scale; **Figure 5C)**. We anticipate an even more pronounced correlation between the actual homologs, namely the lifespans of corpora lutea of pregnancy and nonpregnancy, than is seen between the gestation length and cycle length. We expect this because in many precocial species, gestation is prolonged by non-ovarian sources of progesterone (or accessory corpora lutea), and because our data on nonpregnant cycle refers to the whole cycle rather than just to the luteal phase. Assuming that enough data on the pCL and npCL lifespans can be generated, testing this relationship in the future will inform us whether a coupling between pCL and npCL still imposes a remaining evolutionary constraint to the gestation length increase.

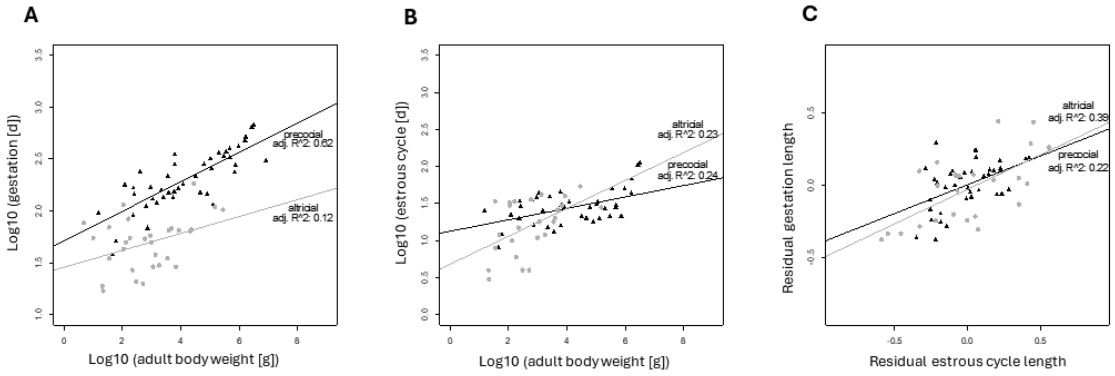

Figure 5. **Regression of body size versus length of nonpregnant and pregnant cycles in precocial and altricial mammals.** Relationship between body size and gestation length **(A)** and body size and estrous cycle length **(B)** on a log10 scale across mammalian taxa (see data in Supplementary Data 3). Pregnancy length and body mass data were sourced from AnAge database (de Magalhães et al. 2024). Ovarian cycles lengths were compiled from several sources (references in the review of the luteal phase and MRP across eutherians). Relationship between the residual pregnant and nonpregnant cycle lengths after regression on the body weight **(C)**. Note that the nonpregnant cycle scales equally strongly with body size in altricial (grey circles) and precocial (black triangles) species. In contrast, gestation length scaling with body size is stronger in precocial species than in altricial species, explaining much of the variation in the former and implying that body size might drive the divergence between precocial and altricial species. After accounting for body size, gestation length correlates well with the nonpregnant cycle length in both cases. All regressions $p<0.05$.

We conducted an ancestral state reconstruction of ovarian cycle and gestation length **(Figure 6)**. Our inference suggests that the ancestral length of the nonpregnant cycle was substantially shorter than the ancestral gestational length (26 days vs. 161 days, respectively). One possibility is that the eutherian ancestor lacked regular, endogenous nonpregnant cycles. It has been suggested that the ancestral type of ovulation is induced, whereas spontaneous ovulation evolved repeatedly within eutherian lineages (Pavlicev and Wagner 2016). The prolongation of the pregnant cycle would have been possible at no cost for the prolonged nonpregnant cycle in cases of induced ovulation. MRP would only become necessary once spontaneous ovulation evolved in major lineages, consistent both with early prolongation of pregnancy, as well as with the evidence for the independent origins of MRP.

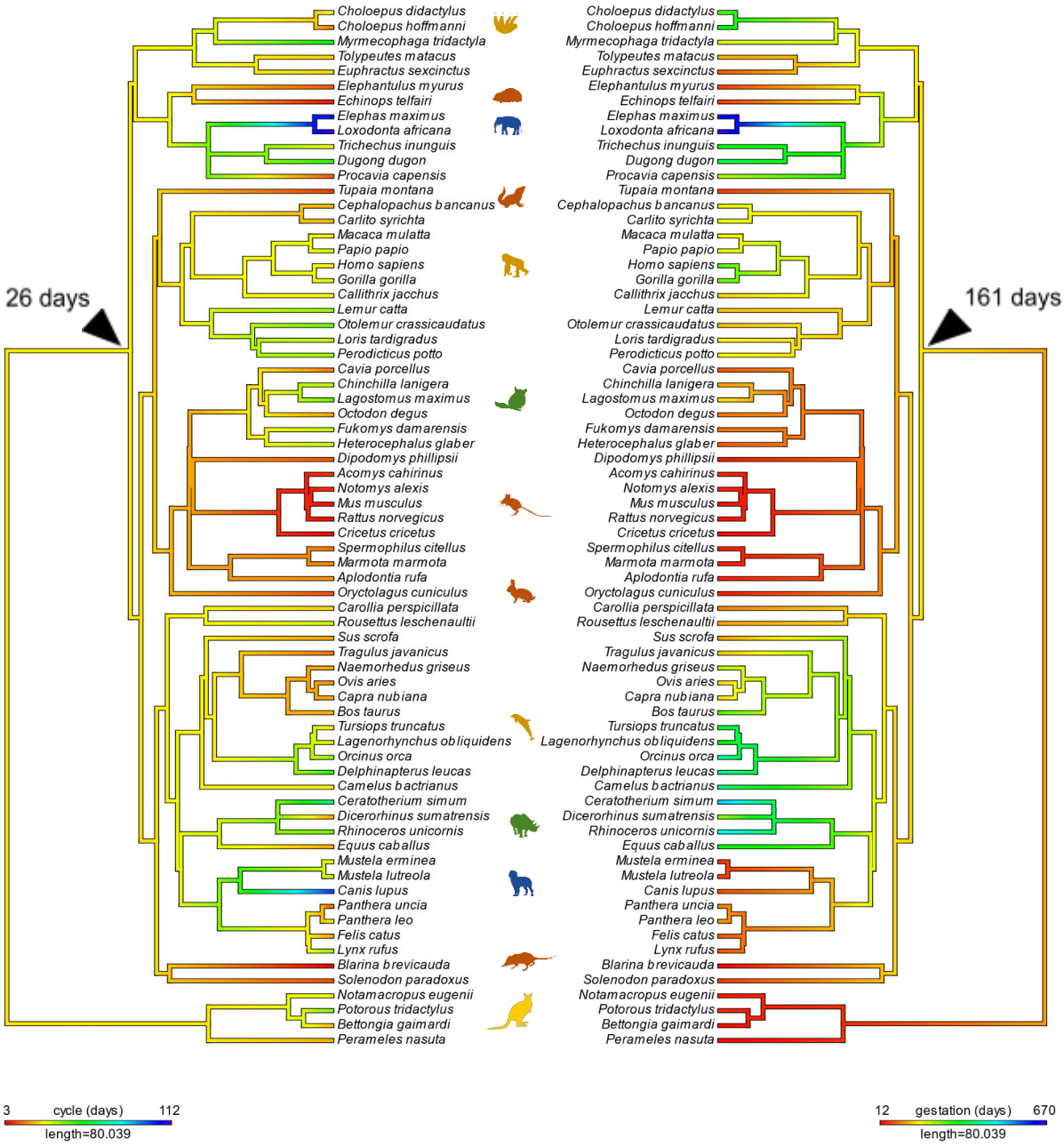

Figure 6. Maximum likelihood ancestral state reconstruction of ovarian cycle and pregnancy lengths (in days) suggest an early and sudden decoupling in terms of cycle length. Plots were generated using the contMap() function in phytools (Revell 2024) and using the phylogenetic tree of timetree (Kumar et al. 2022). Data in Supplementary Data 3. In blue the reconstructed cycle and gestation days for the eutherian common ancestor.

Another possibility is that the ancestral pregnancy length has been systematically overestimated in phylogenetic inference because the origin of MRP introduces a discontinuous change in gestation length and hence extrapolation from species with MRP is not permissible (**Figure 7A and 7B**). Several phylogenetic reconstructions and the fossil data suggest that the ancestral eutherian was of the size of a small rat, but there is less consensus on whether its neonates were altricial or precocial. (altricial: Werneburg et al. 2016, Ferner et al. 2017, precocial: White et al. 2023). Recent fossil

evidence implies that the group with geologically very early size increase already gave birth to precocial neonates (Funston et al. 2022). The independent extensions of gestation will postdate the independent origins of MRP in major lineages if we assume altriciality in the eutherian ancestor. Assuming precociality, on the other hand, entails that an extended gestation length would have occurred already in stem eutherians, possibly in close succession with the origin of implantation. This extension may not have amounted to much, as small body size would require relatively short gestation length even for precocial young.

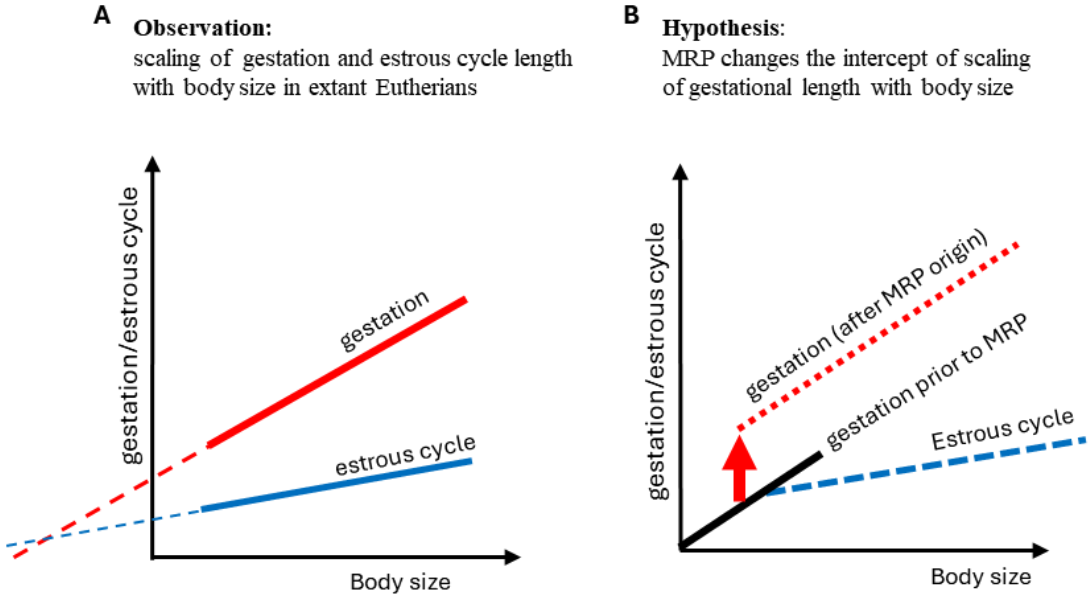

Figure 7. Model explaining the overestimation of gestation length in the ancestral eutherian. A proposed explanation for the inference of relatively long gestational lengths in ancestral eutherian due to the punctuated divergence of gestation from estrous cycle length. Extrapolation is illustrated here relative to body size, as the latter appears to drive gestational length evolution. **A**: The scaling observed in extant eutherians implies a difference in pregnant and nonpregnant cycle lengths at all body sizes. **B**: Hypothetical change of the intercept of gestation length after the origin of MRP (red arrow). The gestation length of an ancestor lacking MRP would be shorter than in extant species with a comparable size. All axes are on log scale. MRP: maternal recognition of pregnancy.

If the pregnant and nonpregnant cycles became decoupled only later, the extension in the stem lineage of Eutheria would prolong both, due to correlated evolution **(Figure 8)**. This scenario thus involves two stages: the first stage consists of an initial extension of gestation and the length of the non-pregnant luteal phase in small-bodied ancestral eutherians. The second stage, driven by an independent selection on body size in all major lineages (Cope 1887, Alroy 1998, Baker et al. 2015), entails the further prolongation of gestation length. In this second stage, the increases in body size intensified the selection for a decoupling between pregnant and non-pregnant cycles. It is in this second stage when the diverse lineage-specific mechanisms for maternal recognition of pregnancy evolved, as well as non-luteal sources of progesterone. Our proposed scenario suggests that the increase in gestation length was mainly driven by the need to developmentally accommodate the increase in body size.

To end with, our scenario implies that the extant derived cases of long-lived npCL (e.g., dog) could have resulted from the extension of the nonpregnant cycle as a consequence of two possible causes **(Figure 8)**. One is a further extension of the pCL, if MRP is lost secondarily due to absence of selection for short npCL. The second could be the advantage of long npCL for pack breeding. In both

cases a long-lived npCL could potentially represent an atavistic regulation. Similarly, ultra-short cycles are likely due to a secondary shortening of the npCL, without changes to gestation length **(Figure 8).** The length of mouse pseudopregnancy would thus reveal the secondarily concealed ancestral luteal phase length. Both derived cases also reveal a persistent underlying correlation between the pregnant and nonpregnant cycle.

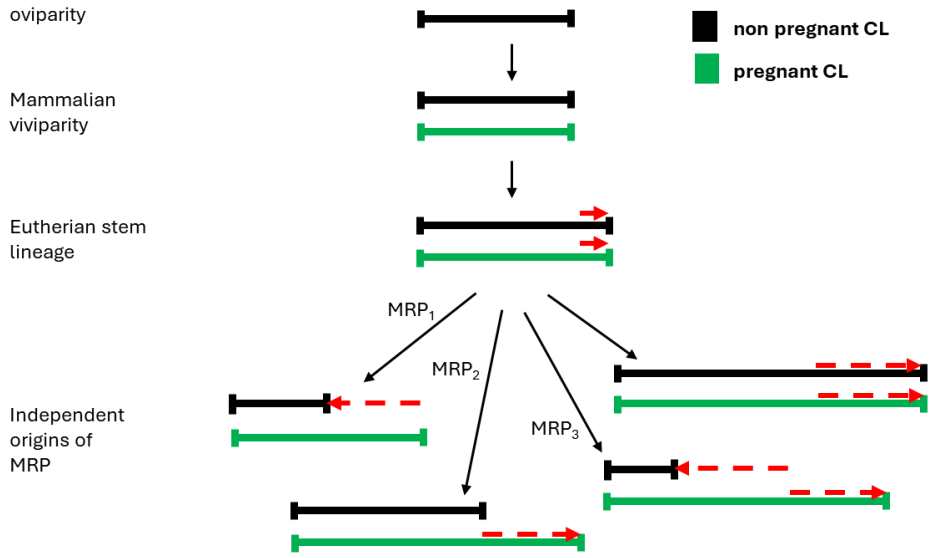

Figure 8. A proposed model for the evolution of the lifespans of pregnant and nonpregnant corpus luteum in eutherians. The model proposes an early co-extension of pregnant and nonpregnant cycle, to be followed by their independent decoupling in major eutherian lineages, by the independent origins of the maternal recognition of pregnancy mechanisms. No claims are made about the sequence of MRP origins here but note that some regimes likely derive from others (for example, MRP(3) may arise from MRP(1) or MRP(2)). MRP: maternal recognition of pregnancy, CL: corpus luteum.

6. Conclusions and prospects

Our aim was to understand when and how the critical innovations of the CL regulation originated in vertebrate evolution, what physiological role they may have played, and what evolutionary consequences they had for female reproduction.

Despite variation in its formation, cell type composition, or the roles of luteal progesterone, the corpus luteum is a homologous vertebrate endocrine structure (Gemmell 1995). Present already in hagfishes, the CL evolved into the context of an existing HP(O) axis, the predecessor of which can be recognized in amphioxus. Early vertebrate genome duplications set the stage for diversifying the factors involved in HPO signaling, specifically pituitary gonadotrophs and steroid sex hormone receptors. Therefore, the origin of the corpus luteum cannot be explained by its current role in eutherian gestation; instead, it predates internal fertilization, and thus any form of ovoviviparity or viviparity. The origin of the gland was associated with the ancestral role of progesterone, likely before progesterone evolved a signaling role via the evolution of its nuclear receptor. We thus suggest that the most plausible early effect of progesterone was to postpone folliculogenesis and delay subsequent ovulations.

We want to emphasize that the evolution of internal fertilization was the critical transition in the evolution of vertebrate reproductive modes. Except for Amniotes, which share a single origin of internal fertilization, all other instances of viviparity origination (elasmobranchs, teleosts, amphibians) are nested in lineages with independently evolved internal fertilization. Consequently, the origins of different instances of viviparity are rooted in different coordination regimes between the ovary and the

reproductive tract and are cases of convergence or parallelism rather than homology, implying that the role of progesterone in these processes likely evolved independently in each group. For these reasons, the inference of mechanisms involved in viviparity across different lineages should be done with much caution (Blackburn 2006). Internal fertilization was the setting in which the new reproductive functions of progesterone first arose. While progesterone is necessary in non-mammalian species for early developmental processes, it is not generally essential for maintaining full-length internal development. The roles that progesterone acquired in non-mammalian reproduction include the ones shared with eutherians, such as the coordination of the ovary with the oviduct and/or the endometrium, but also species-specific ones, like delaying embryonic development or complementing maternal provisioning. Overall, whether progesterone is essential in certain stages of non-mammalian gestation depends on which of these additional, species-specific roles are realized. The increasing regulation of the CL by non-ovarian sources, together with the functional divergence of the post-ovulatory follicle, is a hallmark of eutherian reproduction. We have found no conclusive evidence reported for the existence of such an exogenous regulation extending the pCL lifespan outside placental mammals.

The key innovation in eutherian reproduction is the accommodation of invasive implantation. All eutherians require progesterone from implantation to parturition, even if the serum levels and temporal profiles of progesterone vary widely across species (e.g., from 10 ng/ml in Japanese macaques (Shimizu 2008), >100 ng/ml in mice (Murr et al. 1974), to >200 ng/ml in human). Likely fueled by this requirement, eutherians have evolved multiple but non-redundant ways of controlling CL lifespan or maintaining high progesterone by other means, including blastocyst and decidual signals, accessory corpora lutea, or placental steroidogenesis.

We have argued that a unique characteristic trait in eutherians is the ability to decouple the pregnant and nonpregnant cycles by rescuing the CL only during pregnancy. However, even if maternal recognition of pregnancy (in terms of CL length) is an exclusively eutherian characteristic, it is not a homologous trait among major lineages (mechanisms for MRP differ across the eutherian lineages) and is not universally present across Eutheria. For instance, eutherian species with a long npCL or induced ovulation do not experience the cost of the long nonpregnant cycle. The ancestral reconstruction of the eutherian gestation length likely points to the limits of the phylogenetic method for inferring ancestral traits in isolation from their biological context. The method assumes a continuous change of trait, without substantial punctuated change or novelties. A crucial aspect of our work has been to consider gestation length in the context of its ancestral coupling with the nonpregnant cycle and its coevolution with the body size. This enables us to see that the evolution of the extant gestational lengths involved novelties, namely overcoming the coupling of pregnancy to the nonpregnant cycle (i.e., the origin of MRP), which in addition we argue occurred independently in most major lineages. This decoupling could have led to an abrupt change in the pregnancy length, with little effect on the nonpregnant cycle. In such a situation, the phylogenetic comparative method will overestimate the ancestral pregnancy length. Another aspect of gestational length that makes it a problematic trait to trace, is that it is a composite trait. While implantation is likely homologous, the segments of pregnancy in different eutherian species are likely not and are instead based on independently evolved mechanisms (e.g., a luteal-placental shift in progesterone production at a given gestational point).

An interesting corollary of the proposed model for the evolution of gestation length concerns the evolution of adult mammalian body size. While selection for increased body size has likely driven the extension of gestational length needed to develop a large neonate, the instances of decoupling of the pregnant from nonpregnant cycles were likely the key events enabling the body size increase we see in all major lineages. As pregnancy length must increase with the body size, the lack of decoupling of the nonpregnant luteal phase would mean that in case of a sterile cycle, the next pregnancy would be

substantially delayed, which would be deleterious in most polyestrous species as it lowers the overall reproductive success. The co-distribution of reproductive traits across eutherians does not support conflict narratives depicting a progression towards more fetal control over maternal resources. MRP co-exists with secondarily non-invasive placentation, such as in ungulates, or with the lack of a luteal-placental shift, such as in proboscidea species, where the accessory CL prolonging pregnancy are in fact maternal structures. Rather than by mother-offspring conflict, the origin of MRP and extended pregnancy is likely driven by evolution of an increased body size.

Finally, we would like to note that there are many aspects that remain unaddressed in our scenario, in part due to the lack of data for many species in the wild, and in part due to the lack of attention to the nonpregnant cycles, or to the lifespan of the CL. In addition, gestational length is certainly affected by further factors that we did not consider here, such as litter size or growth rate. Another important aspect that remains unaddressed in this review, but may reveal further, past or present, constraints, are the roles that reproductive factors played in the development and function of the male reproductive system. As male and female reproductive functions may often be coupled in the evolutionary process (e.g., pituitary hormones also regulate the function of male gonads), evolutionary explanations may require considering the biology of both sexes.

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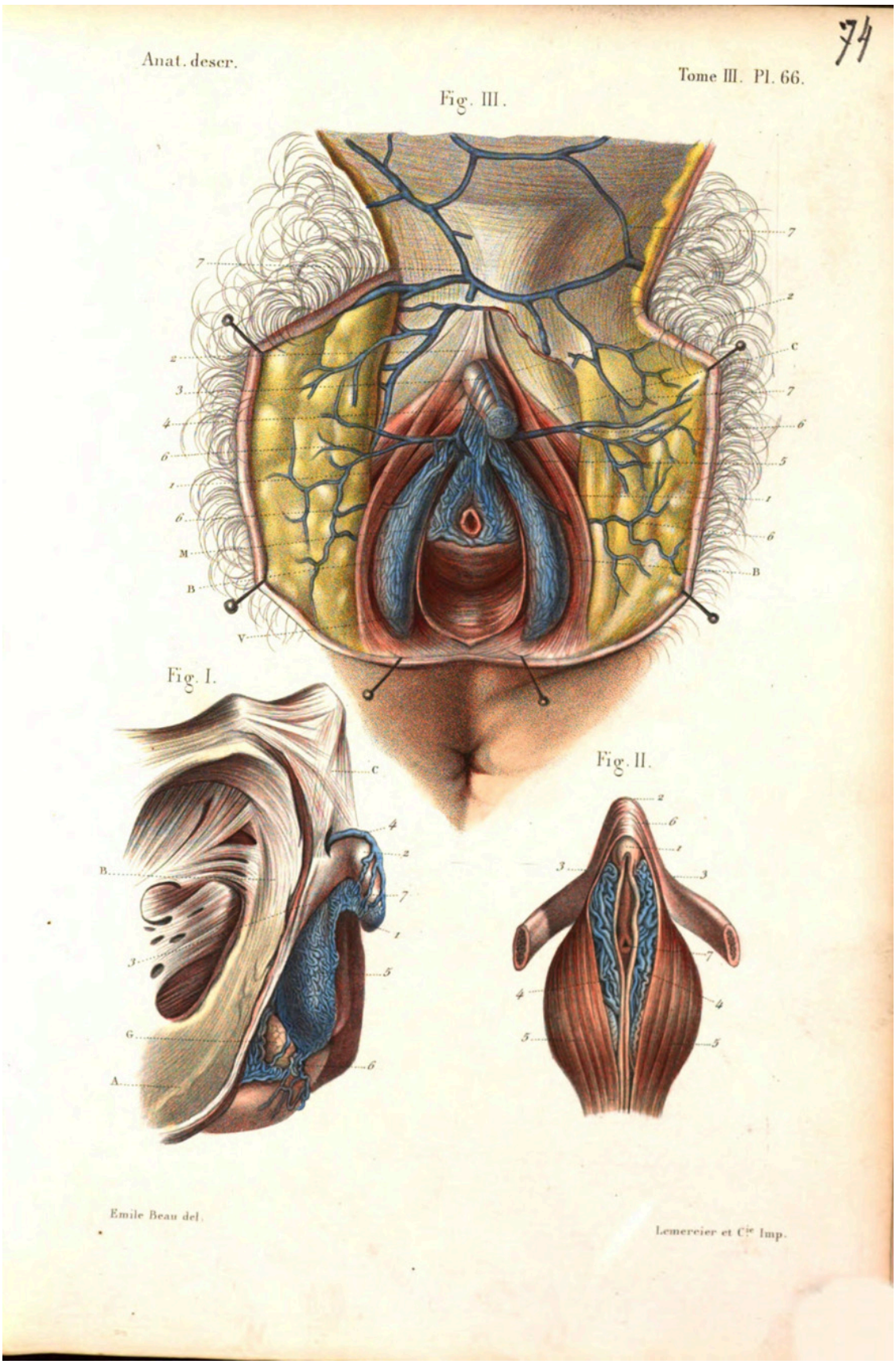
Supplementary Data 1. Data of reproductive characters							
Species	**Common Name**	**Order**	**CL_type**	**Placental_P4**	**Ovulation**	**Prec/altr**	**Invasiveness**
Dasypus_novemcinctus	Nine-banded armadillo	Cingulata		No	Spontaneous	prec	Hemochorial
Euphractus_sexcinctus	Six-banded armadillo	Cingulata	Short-lived	No	Spontaneous	altr	Hemochorial
Tolypeutes_matacus	Three-banded armadillo	Cingulata	Short-lived	No	Spontaneous	prec	Hemochorial
Myrmecophaga_tridactyla	Giant anteater	Cingulata	Short-lived		Spontaneous	altr	Hemochorial
Bradypus_torquatus	Maned three-toed sloth	Cingulata	Short-lived		Spontaneous	prec	Endotheliochorial
Choloepus_didactylus	Southern two-toed sloth	Cingulata	Short-lived		Spontaneous	prec	Endotheliochorial
Choloepus_hoffmanni	Two-toed sloth	Cingulata	Short-lived		Spontaneous	prec	Endotheliochorial
Loxodonta_africana	African savanna elephant	Proboscidea	Short-lived	No	Spontaneous	prec	Endotheliochorial
Elephas_maximus	Asian elephant	Proboscidea	Short-lived	No	Spontaneous	prec	Endotheliochorial
Dugong_dugon	Sea cow	Sirenia	Short-lived		Spontaneous	prec	Endotheliochorial
Trichechus_inunguis	Amazon manatee	Sirenia	Short-lived		Spontaneous	prec	Endotheliochorial
Procavia_capensis	Cape rock hyrax	Hyracoidea	Short-lived		Spontaneous	prec	Hemochorial
Tenrec_ecaudatus	Tailess tenrec	Macroscelidea			Induced	altr	Hemochorial
Echinops_telfairi	Small madagascar hedgehog	Macroscelidea	Short-lived		Induced	altr	Hemochorial
Elephantulus_myurus	Eastern rock elephant shrew	Afrosoricida	Short-lived	No	Spontaneous	prec	Hemochorial
Bos_taurus	Cattle	Artiodactyla	Short-lived	Yes	Spontaneous	prec	Epitheliochorial
Sus_scrofa	Pig	Artiodactyla	Short-lived	Yes	Spontaneous	prec	Epitheliochorial
Ovis_aries	Sheep	Artiodactyla	Short-lived	Yes	Spontaneous	prec	Epitheliochorial
Capra_nubiana	Goat	Artiodactyla	Short-lived	No	Spontaneous	prec	Epitheliochorial
Camelus_bactrianus	Bactrian camel	Artiodactyla	Short-lived	Yes	Induced	prec	Epitheliochorial
Naemorhedus_griseus	Chinese goral	Artiodactyla	Short-lived		Spontaneous	prec	Epitheliochorial
Tragulus_javanicus	Lesser Malay mouse deer	Artiodactyla	Short-lived		Spontaneous	prec	Epitheliochorial
Equus_caballus	Horse	Perissodactyla	Short-lived	Yes	Spontaneous	prec	Epitheliochorial
Rhinoceros_unicornis	Indian rhinoceros	Perissodactyla	Short-lived	Yes	Spontaneous	prec	Epitheliochorial
Ceratotherium_simum	White rhinoceros	Perissodactyla	Short-lived	Yes	Spontaneous	prec	Epitheliochorial
Dicerorhinus_sumatrensis	Asian rhinoceros	Perissodactyla	Short-lived	Yes	Induced	prec	Epitheliochorial
Delphinapterus_leucas	Beluga whale	Cetacea	Short-lived		Both	prec	Epitheliochorial
Tursiops_truncatus	Atlantic bottlenose dolphin	Cetacea	Short-lived		Spontaneous	prec	Epitheliochorial
Orcinus_orca	Orca whale	Cetacea	Short-lived		Spontaneous	prec	Epitheliochorial
Lagenorhynchus_obliquidens	Pacific white-sided dolphins	Cetacea	Short-lived		Spontaneous	prec	Epitheliochorial
Balaenoptera_bonaerensis	Minke whale	Cetacea	Short-lived	Yes	Spontaneous	prec	Epitheliochorial
Nycticeinops_schlieffeni	Schlieffen's bat	Chiroptera	Short-lived	No	Spontaneous	altr	Hemochorial
Rousettus_leschenaultii	Fulvous fruit bat	Chiroptera	Short-lived		Spontaneous	altr	Hemochorial
Carollia_perspicillata	Short-tailed Fruit Bat	Chiroptera	Short-lived		Spontaneous	prec	Hemochorial
Miniopterus_natalensis	Natal clinging bat	Chiroptera	Short-lived	Yes	Spontaneous	altr	Hemochorial
Myotis_lucifugus	Little brown bat	Chiroptera	Short-lived	Yes	Spontaneous	altr	Hemochorial
Mustela_lutreola	European Mink	Carnivora	Short-lived		Spontaneous	altr	Endotheliochorial
Mustela_erminea	Ermine	Carnivora	Short-lived		Spontaneous	altr	Endotheliochorial
Cystophora_cristata	Hooded seal	Carnivora		No	Spontaneous	prec	Endotheliochorial
Ursus_arctos	Brown bears	Carnivora		No	Both	altr	Endotheliochorial
Canis_lupus	Domestic dog	Carnivora	Long-lived	No	Spontaneous	altr	Endotheliochorial
Chrysocyon_brachyurus	Maned wolf	Carnivora	Long-lived	No	Induced	altr	Endotheliochorial
Panthera_leo	Lion	Carnivora	Short-lived		Both	altr	Endotheliochorial
Panthera_uncia	Snow leopard	Carnivora	Short-lived		Induced	altr	Endotheliochorial
Felis_catus	Domestic cat	Carnivora	Short-lived	Yes	Both	altr	Endotheliochorial
Lynx_lynx	Eurasian lynx	Carnivora	Short-lived	No	Both	altr	Endotheliochorial
Lynx_rufus	Bobcat	Carnivora	Short-lived	Yes	Both	altr	Endotheliochorial
Blarina_brevicauda	Short-tailed shrew	Eulipotyphla	Ultra-short	No	Induced	altr	Endotheliochorial
Solenodon_paradoxus	Hispaniolan solenodon	Eulipotyphla	Short-lived	No		altr	Endotheliochorial
Oryctolagus_cuniculus	European rabbit	Lagomorpha	Short-lived	Yes	Induced	altr	Hemochorial
Cavia_porcellus	Guinea pig	Rodents	Short-lived	Yes	Spontaneous	prec	Hemochorial
Lagostomus_maximus	Plains viscacha	Rodents	Short-lived	No	Spontaneous	prec	Hemochorial
Chinchilla_lanigera	Long-tailed chinchilla	Rodents	Short-lived		Spontaneous	prec	Hemochorial
Octodon_degus	Defu	Rodents	Short-lived			prec	Hemochorial
Heterocephalus_glaber	Naked mole rat	Rodents	Short-lived		Spontaneous	altr	Hemochorial
Fukomys_damarensis	Damaraland mole-rats	Rodents	Short-lived		Spontaneous	altr	Hemochorial
Aplodontia_rufa	Arena Mountain Beaver	Rodents	Short-lived		Spontaneous	altr	Hemochorial
Marmota_marmota	Alpine marmot	Rodents	Short-lived			altr	Hemochorial
Spermophilus_citellus	European ground squirrel	Rodents	Short-lived		Spontaneous	altr	Hemochorial

Dipodomys_phillipsii	Kangaroo rat	Rodents	Short-lived		Spontaneous	altr	Hemochorial
Mus_musculus	Mice	Rodents	Ultra-short	No	Spontaneous	altr	Hemochorial
Cricetus_cricetus	Hamster	Rodents	Ultra-short	No	Spontaneous	altr	Hemochorial
Rattus_norvegicus	Black rat	Rodents	Ultra-short	No	Spontaneous	altr	Hemochorial
Notomys_alexis	Spinifex hopping mouse	Rodents	Short-lived		Spontaneous	altr	Hemochorial
Acomys_cahirinus	African spiny mouse	Rodents	Short-lived	No	Spontaneous	prec	Hemochorial
Perodicticus_potto	West African potto	Primate	Short-lived		Spontaneous	prec	Epitheliochorial
Otolemur_crassicaudatus	Brown greater galago	Primate	Short-lived		Spontaneous	prec	Epitheliochorial
Carlito_syrichta	Philippines tarsier	Primate	Short-lived		Spontaneous	prec	Hemochorial
Lemur_catta	Ring-tailed lemur	Primate	Short-lived		Spontaneous	prec	Epitheliochorial
Callithrix_jacchus	Common marmoset	Primate	Short-lived	Yes	Spontaneous	prec	Hemochorial
Papio_papio	Baboon	Primate	Short-lived	Yes	Spontaneous	prec	Hemochorial
Macaca_mulatta	Rhesus macaque	Primate	Short-lived	Yes	Spontaneous	prec	Hemochorial
Homo_sapiens	Humans	Primate	Short-lived	Yes	Spontaneous	prec	Hemochorial
Loris_tardigradus	Slender loris	Primate	Short-lived		Spontaneous	prec	Epitheliochorial
Tupaia_montana	Tree shrew	Scadentia	Short-lived		Induced	altr	Endotheliochorial
Cephalopachus_bancanus	Tarsier	Primate	Short-lived		Spontaneous	prec	Hemochorial
Gorilla_gorilla	Gorilla	Primate	Short-lived	Yes	Spontaneous	prec	Hemochorial

Supplementary Data 2. Types of corpus luteum and MRP					
Species	**Common Name**	**Order**	**CL_type**	**MRP**	**Type**
Dasypus_novemcinctus	Nine-banded armadillo	Cingulata			
Euphractus_sexcinctus	Six-banded armadillo	Cingulata	Short-lived		
Tolypeutes_matacus	Three-banded armadillo	Cingulata	Short-lived		
Myrmecophaga_tridactyla	Giant anteater	Cingulata	Short-lived		
Bradypus_torquatus	Maned three-toed sloth	Cingulata	Short-lived		
Choloepus_didactylus	Southern two-toed sloth	Cingulata	Short-lived		
Choloepus_hoffmanni	Two-toed sloth	Cingulata	Short-lived		
Loxodonta_africana	African savanna elephant	Proboscidea	Short-lived	PL	Luteotrophic
Elephas_maximus	Asian elephant	Proboscidea	Short-lived	PL	Luteotrophic
Dugong_dugon	Sea cow	Sirenia	Short-lived		
Trichechus_inunguis	Amazon manatee	Sirenia	Short-lived		
Procavia_capensis	Cape rock hyrax	Hyracoidea	Short-lived		
Tenrec_ecaudatus	Tailess tenrec	Macroscelidea			
Echinops_telfairi	Small madagascar hedgehog	Macroscelidea	Short-lived		
Elephantulus_myurus	Eastern rock elephant shrew	Afrosoricida	Short-lived		
Bos_taurus	Cattle	Artiodactyla	Short-lived	IFNT	Anti-luteolytic
Sus_scrofa	Pig	Artiodactyla	Short-lived	E2	Anti-luteolytic
Ovis_aries	Sheep	Artiodactyla	Short-lived	IFNT	Anti-luteolytic
Capra_nubiana	Goat	Artiodactyla	Short-lived	IFNT	Anti-luteolytic
Camelus_bactrianus	Bactrian camel	Artiodactyla	Short-lived	E2	Anti-luteolytic
Naemorhedus_griseus	Chinese goral	Artiodactyla	Short-lived		
Tragulus_javanicus	Lesser Malay mouse deer	Artiodactyla	Short-lived		
Equus_caballus	Horse	Perissodactyla	Short-lived		
Rhinoceros_unicornis	Indian rhinoceros	Perissodactyla	Short-lived		
Ceratotherium_simum	White rhinoceros	Perissodactyla	Short-lived		
Dicerorhinus_sumatrensis	Asian rhinoceros	Perissodactyla	Short-lived		
Delphinapterus_leucas	Beluga whale	Cetacea	Short-lived		
Tursiops_truncatus	Atlantic bottlenose dolphin	Cetacea	Short-lived		
Orcinus_orca	Orca whale	Cetacea	Short-lived		
Lagenorhynchus_obliquidens	Pacific white-sided dolphins	Cetacea	Short-lived		
Balaenoptera_bonaerensis	Minke whale	Cetacea	Short-lived		
Nycticeinops_schlieffeni	Schlieffen's bat	Chiroptera	Short-lived		
Rousettus_leschenaultii	Fulvous fruit bat	Chiroptera	Short-lived		
Carollia_perspicillata	Short-tailed Fruit Bat	Chiroptera	Short-lived		
Miniopterus_natalensis	Natal clinging bat	Chiroptera	Short-lived		
Myotis_lucifugus	Little brown bat	Chiroptera	Short-lived		
Mustela_lutreola	European Mink	Carnivora	Short-lived		
Mustela_erminea	Ermine	Carnivora	Short-lived		
Cystophora_cristata	Hooded seal	Carnivora			
Ursus_arctos	Brown bears	Carnivora			
Canis_lupus	Domestic dog	Carnivora	Long-lived	Absent	
Chrysocyon_brachyurus	Maned wolf	Carnivora	Long-lived	Absent	
Panthera_leo	Lion	Carnivora	Short-lived		
Panthera_uncia	Snow leopard	Carnivora	Short-lived		
Felis_catus	Domestic cat	Carnivora	Short-lived		
Lynx_lynx	Eurasian lynx	Carnivora	Short-lived		
Lynx_rufus	Bobcat	Carnivora	Short-lived		
Blarina_brevicauda	Short-tailed shrew	Eulipotyphla	Ultra-short		
Solenodon_paradoxus	Hispaniolan solenodon	Eulipotyphla	Short-lived		
Oryctolagus_cuniculus	European rabbit	Lagomorpha	Short-lived		

Cavia_porcellus	Guinea pig	Rodents	Short-lived		
Lagostomus_maximus	Plains viscacha	Rodents	Short-lived		
Chinchilla_lanigera	Long-tailed chinchilla	Rodents	Short-lived		
Octodon_degus	Defu	Rodents	Short-lived		
Heterocephalus_glaber	Naked mole rat	Rodents	Short-lived		
Fukomys_damarensis	Damaraland mole-rats	Rodents	Short-lived		
Aplodontia_rufa	Arena Mountain Beaver	Rodents	Short-lived		
Marmota_marmota	Alpine marmot	Rodents	Short-lived		
Spermophilus_citellus	European ground squirrel	Rodents	Short-lived		
Dipodomys_phillipsii	Kangaroo rat	Rodents	Short-lived	Maternal_PRL	Luteotrophic
Mus_musculus	Mice	Rodents	Ultra-short	Maternal_PRL	Luteotrophic
Cricetus_cricetus	Hamster	Rodents	Ultra-short	Maternal_PRL	Luteotrophic
Rattus_norvegicus	Black rat	Rodents	Ultra-short	Maternal_PRL	Luteotrophic
Notomys_alexis	Spinifex hopping mouse	Rodents	Short-lived		
Acomys_cahirinus	African spiny mouse	Rodents	Short-lived		
Perodicticus_potto	West African potto	Primate	Short-lived		
Otolemur_crassicaudatus	Brown greater galago	Primate	Short-lived		
Carlito_syrichta	Philippines tarsier	Primate	Short-lived		
Lemur_catta	Ring-tailed lemur	Primate	Short-lived		
Callithrix_jacchus	Common marmoset	Primate	Short-lived	CG	Luteotrophic
Papio_papio	Baboon	Primate	Short-lived	CG	Luteotrophic
Macaca_mulatta	Rhesus macaque	Primate	Short-lived	CG	Luteotrophic
Homo_sapiens	Humans	Primate	Short-lived	CG	Luteotrophic
Loris_tardigradus	Slender loris	Primate	Short-lived		
Tupaia_montana	Tree shrew	Scadentia	Short-lived		
Cephalopachus_bancanus	Tarsier	Primate	Short-lived		
Gorilla_gorilla	Gorilla	Primate	Short-lived	CG	Luteotrophic

Supplementary Data 3. Cycle and gestation day values for regression plots and phylogenetic analysis									
Species	**Common Name**	**Order**	**Superorder**	**Cycle_length**	**Gestation_length_d_AnAge**	**wtcorrCL**	**wtcorrPrg**	**Body_mass_g_AnAge**	**Prec/altr**
Dasypus_novemcinctus	Nine-banded armadillo	Cingulata	Xenarthra	NA	133	NA	2.1803	5500	prec
Euphractus_sexcinctus	Six-banded armadillo	Cingulata	Xenarthra	26	68	1.2411	1.7661	4850	altr
Tolypeutes_matacus	Three-banded armadillo	Cingulata	Xenarthra	25	120	1.4715	2.2155	1500	prec
Myrmecophaga_tridactyla	Giant anteater	Cingulata	Xenarthra	54	184	1.4139	2.1359	28500	altr
Bradypus_torquatus	Maned three-toed sloth	Cingulata	Xenarthra	NA	150	NA	2.2541	3875	prec
Choloepus_didactylus	Southern two-toed sloth	Cingulata	Xenarthra	30	279	1.503	2.4942	6250	prec
Choloepus_hoffmanni	Two-toed sloth	Cingulata	Xenarthra	16	350	1.23	2.5926	6250	prec
Loxodonta_africana	African savanna elephant	Proboscidea	Afrotheria	112	670	1.8667	2.4914	3175000	prec
Elephas_maximus	Asian elephant	Proboscidea	Afrotheria	105	644	1.8441	2.4842	2700000	prec
Dugong_dugon	Sea cow	Sirenia	Afrotheria	50	374	1.5893	2.3721	360000	prec
Trichechus_inunguis	Amazon manatee	Sirenia	Afrotheria	30	328	1.3578	2.2974	480000	prec
Procavia_capensis	Cape rock hyrax	Hyracoidea	Afrotheria	13	215	1.1582	2.4149	3600	prec
Tenrec_ecaudatus	Tailess tenrec	Macroscelidea	Afrotheria	NA	58	NA	1.7566	900	altr
Echinops_telfairi	Small madagascar hedgehog	Macroscelidea	Afrotheria	6	55	0.8732	1.7904	180	altr
Elephantulus_myurus	Eastern rock elephant shrew	Afrosoricida	Afrotheria	12	51	1.2604	2.0419	60	prec
Bos_taurus	Cattle	Artiodactyla	Laurasiatheria	21	277	1.188	2.1966	750000	prec
Sus_scrofa	Pig	Artiodactyla	Laurasiatheria	20	115	1.2254	1.9226	130000	prec
Ovis_aries	Sheep	Artiodactyla	Laurasiatheria	18	146	1.1959	2.0561	80000	prec
Capra_nubiana	Goat	Artiodactyla	Laurasiatheria	20	150	1.2599	2.1015	46250	prec
Camelus_bactrianus	Bactrian camel	Artiodactyla	Laurasiatheria	28	395	1.3282	2.3788	475000	prec
Naemorhedus_griseus	Chinese goral	Artiodactyla	Laurasiatheria	21	215	1.2934	2.2806	32000	prec
Tragulus_javanicus	Lesser Malay mouse deer	Artiodactyla	Laurasiatheria	15	159	1.2368	2.3142	2200	prec
Equus_caballus	Horse	Perissodactyla	Laurasiatheria	21	337	1.2186	2.3381	300000	prec
Rhinoceros_unicornis	Indian rhinoceros	Perissodactyla	Laurasiatheria	43	479	1.4738	2.3877	1602330	prec
Ceratotherium_simum	White rhinoceros	Perissodactyla	Laurasiatheria	68	515	1.6709	2.4156	1700000	prec
Dicerorhinus_sumatrensis	Asian rhinoceros	Perissodactyla	Laurasiatheria	21	236	1.1903	2.1312	700000	prec
Delphinapterus_leucas	Beluga whale	Cetacea	Laurasiatheria	50	416	1.5568	2.3586	950000	prec
Tursiops_truncatus	Atlantic bottlenose dolphin	Cetacea	Laurasiatheria	27	365	1.3413	2.3977	200000	prec
Orcinus_orca	Orca whale	Cetacea	Laurasiatheria	40	510			3500000	prec
Lagenorhynchus_obliquidens	Pacific white-sided dolphins	Cetacea	Laurasiatheria	31	320	1.4235	2.3814	103000	prec
Balaenoptera_bonaerensis	Minke whale	Cetacea	Laurasiatheria	NA	305	NA	2.0909	8250000	prec
Nycticeinops_schlieffeni	Schlieffen's bat	Chiroptera	Laurasiatheria	NA	77	NA	2.0653	4.7	altr
Rousettus_leschenaultii	Fulvous fruit bat	Chiroptera	Laurasiatheria	33	115			108.25	altr
Carollia_perspicillata	Short-tailed Fruit Bat	Chiroptera	Laurasiatheria	25	95	1.6255	2.3974	15	prec
Miniopterus_natalensis	Natal clinging bat	Chiroptera	Laurasiatheria	NA	NA	NA	NA	NA	altr
Myotis_lucifugus	Little brown bat	Chiroptera	Laurasiatheria	NA	55	NA	1.8925	10	altr
Mustela_lutreola	European Mink	Carnivora	Laurasiatheria	36	54	1.5544	1.7405	590	altr
Mustela_erminea	Ermine	Carnivora	Laurasiatheria	30	43	1.6121	1.7008	110.3	altr
Cystophora_cristata	Hooded seal	Carnivora	Laurasiatheria	NA	240	NA	2.5971	405	prec
Ursus_arctos	Brown bears	Carnivora	Laurasiatheria	NA	103	NA	1.8035	277500	altr
Canis_lupus	Domestic dog	Carnivora	Laurasiatheria	100	62			26625	altr
Chrysocyon_brachyurus	Maned wolf	Carnivora	Laurasiatheria	NA	64	NA	1.6872	21500	altr
Panthera_leo	Lion	Carnivora	Laurasiatheria	28	109	0.9931	1.8498	150000	altr
Panthera_uncia	Snow leopard	Carnivora	Laurasiatheria	NA	NA	NA	NA	50000	altr
Felis_catus	Domestic cat	Carnivora	Laurasiatheria	20	65	1.1449	1.7542	3900	altr
Lynx_lynx	Eurasian lynx	Carnivora	Laurasiatheria	NA	66	NA	1.6982	23000	altr
Lynx_rufus	Bobcat	Carnivora	Laurasiatheria	40	65	1.3814	1.7263	8600	altr
Blarina_brevicauda	Short-tailed shrew	Eulipotyphla	Laurasiatheria	3	17	0.7452	1.3554	21.6	altr
Solenodon_paradoxus	Hispaniolan solenodon	Eulipotyphla	Laurasiatheria	11	50	0.9964	1.6884	1000	altr
Oryctolagus_cuniculus	European rabbit	Lagomorpha	Euarchontoglires	12	30	0.9862	1.4458	1800	altr
Cavia_porcellus	Guinea pig	Rodents	Euarchontoglires	16	68	1.3019	2.0133	728	prec
Lagostomus_maximus	Plains viscacha	Rodents	Euarchontoglires	45	154	1.6804	2.2386	6000	prec
Chinchilla_lanigera	Long-tailed chinchilla	Rodents	Euarchontoglires	35	111	1.646	2.2338	642.5	prec
Octodon_degus	Defu	Rodents	Euarchontoglires	20	90	1.4366	2.2046	235	prec
Heterocephalus_glaber	Naked mole rat	Rodents	Euarchontoglires	34	70	1.7602	1.953	35	altr
Fukomys_damarensis	Damaraland mole-rats	Rodents	Euarchontoglires	34	84	1.6361	1.9785	160	altr
Aplodontia_rufa	Arena Mountain Beaver	Rodents	Euarchontoglires	18	29	1.2007	1.4477	1125	altr
Marmota_marmota	Alpine marmot	Rodents	Euarchontoglires	18	35	1.108	1.4892	3500	altr
Spermophilus_citellus	European ground squirrel	Rodents	Euarchontoglires	15	27	1.2558	1.4748	217	altr
Dipodomys_phillipsii	Kangaroo rat	Rodents	Euarchontoglires	12	NA	1.2821	NA	48	altr
Mus_musculus	Mice	Rodents	Euarchontoglires	4	19	0.8744	1.4055	20.5	altr
Cricetus_cricetus	Hamster	Rodents	Euarchontoglires	4	20	0.6126	1.3145	506.7	altr
Rattus_norvegicus	Black rat	Rodents	Euarchontoglires	4	21	0.6554	1.3542	300	altr
Notomys_alexis	Spinifex hopping mouse	Rodents	Euarchontoglires	8	35	1.1318	1.6519	35	altr
Acomys_cahirinus	African spiny mouse	Rodents	Euarchontoglires	8	38	1.0938	1.9316	45.2	prec
Perodicticus_potto	West African potto	Primate	Euarchontoglires	38	170	1.6697	2.3969	920	prec
Otolemur_crassicaudatus	Brown greater galago	Primate	Euarchontoglires	44	130	1.7275	2.2697	1095	prec
Carlito_syrichta	Philippines tarsier	Primate	Euarchontoglires	22	179	1.5007	2.545	119.2	prec
Lemur_catta	Ring-tailed lemur	Primate	Euarchontoglires	39	135	1.6468	2.2339	2555	prec
Callithrix_jacchus	Common marmoset	Primate	Euarchontoglires	29	144	1.5952	2.4037	255.2	prec
Papio_papio	Baboon	Primate	Euarchontoglires	33	180	1.5225	2.2637	12000	prec
Macaca_mulatta	Rhesus macaque	Primate	Euarchontoglires	28	165	1.4638	2.2491	8235	prec
Homo_sapiens	Humans	Primate	Euarchontoglires	28	280	1.3962	2.3546	62035	prec
Loris_tardigradus	Slender loris	Primate	Euarchontoglires	34	166	1.6666	2.4697	238.2	prec
Tupaia_montana	Tree shrew	Scadentia	Euarchontoglires	10	50	1.1216	1.7605	130	altr
Cephalopachus_bancanus	Tarsier	Primate	Euarchontoglires	22	178	1.4998	2.5409	122.5	prec
Gorilla_gorilla	Gorilla	Primate	Euarchontoglires	33	256	1.4474	2.2785	113500	prec
Potorous_tridactylus	Long-nosed potoroo	Diprotodontia	Australidelphia	42	38	1.5538	1.5586	1350	altr
Notamacropus_eugenii	Tammar wallaby	Diprotodontia	Australidelphia	30	29	1.2745	1.3836	6900	altr
Bettongia_gaimardi	Eastern betong	Diprotodontia	Australidelphia	19.5	21			1200	altr
Perameles_nasuta	Long-nosed bandicoot	Peramelemorphia	Australidelphia	21	12.5			760	altr



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RESEARCH ARTICLE

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The female orgasm and the homology concept in evolutionary biology

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Abstract

The definition of homology and its application to reproductive structures, external genitalia, and the physiology of sexual pleasure has a tortuous history. While nowadays there is a consensus on the developmental homology of genital and reproductive systems, there is no agreement on the physiological translation, or the evolutionary origination and roles, of these structural correspondences and their divergent histories. This paper analyzes the impact of evolutionary perspectives on the homology concept as applied to the female orgasm, and their consequences for the biological and social understanding of female sexuality and reproduction. After a survey of the history of pre-evolutionary biomedical views on sexual difference and sexual pleasure, we examine how the concept of sexual homology was shaped in the new phylogenetic framework of the late 19th century. We then analyse the debates on the anatomical locus of female pleasure at the crossroads of theories of sexual evolution and new scientific discourses in psychoanalysis and sex studies. Moving back to evolutionary biology, we explore the consequences of neglecting homology in adaptive explanations of the female orgasm. The last two sections investigate the role played by different articulations of the homology concept in evolutionary developmental explanations of the origin and evolution of the female orgasm. These include the role of sexual, developmental homology in the byproduct hypothesis, and a more recent hypothesis where a phylogenetic, physiological concept of homology is used to account for the origination of the female orgasm. We conclude with a brief discussion on the social implications for the understanding of female pleasure derived from these different homology frameworks.

KEYWORDS

clitoris, evo-devo, female orgasm, history of sexuality, homology

1 | INTRODUCTION

Scientific controversies on the female orgasm condense how gender biases have shaped research on female anatomy and physiology, and how biological sciences, in turn, have both constrained and enabled new narratives on human sexuality. Far from being a resolved topic, the biomedical representation and explanation of the female orgasm is a matter of lively debate in various biological disciplines concerned with the study of sexuality, including medical anatomy, animal endocrinology and neurophysiology, and evolutionary biology.

Previous studies by gender and sexuality scholars have documented various episodes of the history of ideas about female genitalia and the physiology of pleasure (e.g., Laqueur, 1986; Lloyd, 2005; Moore, 2018, 2021; Tuana, 2004). This paper aims to complement these works by looking at how changing notions of homology have been used in evolutionary studies of female pleasure in the context of different societal assumptions and expectations on female sexuality.

Textbook depictions of male and female urogenital systems in mammals agree in presenting them as biological homologs (e.g., Carlson, 2018, pp. 394–401). In the early stages, mammalian embryos have indifferent structures that develop into male or female derivatives. From an undifferentiated sexual duct system, it is the paramesonephric (Müllerian) ducts that further develop in the case of a female embryo. In males, it is the mesonephric (Wolffian) ducts that give rise to the structures encompassing the epididymis, seminal vesicles, and ejaculatory duct. The genital ridge develops into ovaries or testes, the primordial germ cells into ova or spermatozoa, and the sex cords into granulosa or Sertoli cells. The external genitalia also develop from an undifferentiated condition: the genital tubercle develops into the glans penis or the clitoris, the urogenital fold becomes the spongy urethra in males and the labia minora in females, and the genital swellings give rise to the scrotum in males and the labia majora in females.

The definition of homology and its application to reproductive structures, external genitalia, and the physiology of sexual pleasure has a tortuous history, and while nowadays there is a wide consensus on the developmental homology of genital and reproductive structures, there is no agreement on the physiological translation, neither on the evolutionary origination and roles, of these structural homologies. This article analyzes the impact of the comparative perspective in biomedical representations of female sexuality and female reproduction since the 19th century to contemporary debates on the role and nature of the female orgasm. More specifically, we dig into evolutionary perspectives of homology and their consequences for the biological and social understanding of female sexuality and reproduction.

The structure of the article will be as follows. In Section 2, we survey the history of pre-evolutionary biomedical views on sexual difference and sexual pleasure. Section 3 refers to the shaping of the concept of sexual homology by comparative anatomists in a new phylogenetic framework towards the end of the 19th century. Section 4 analyses debates on the anatomical locus of female pleasure at the crossroads of theories of sexual evolution and new scientific discourses in psychoanalysis and sex studies. Section 5 moves back to evolutionary biology and examines the consequences of neglecting homology in adaptive explanations of the female orgasm. The last two sections investigate the core role played by different articulations of the homology concept in evolutionary developmental explanations of the origin and evolution of the female orgasm. Section 6 looks at the role of sexual homology in articulating the byproduct hypothesis. Section 7 examines a more recent hypothesis where a phylogenetic, physiological concept of homology, is applied to account for the origination of the female orgasm. We conclude with a brief discussion on the societal implications for understanding female pleasure derived from these different homology frameworks.

2 | EROTIC HOMOLOGY AND REPRODUCTIVE HOMOLOGY IN PRE-EVOLUTIONARY TIMES

Before the rise of comparative anatomy in the 19th century, the recognition of identity relationships between individual organisms relied either on analogical reasoning, or on intuitive understandings of "sameness," rather than on any formal criteria of homology. Nonetheless, since Aristotle, comparative studies used different informal criteria based on the number, relative size, and position of organs, to characterize the "unity in diversity" of animal form. Identity relationships included those holding between the sexes, what we refer today as sexual homology, namely an instance of serial homology that captures the relation of identity between traits belonging to the two sexes of the same species (Fusco, 2022). Different conceptualizations of sexual homology constituted the conceptual framework where biomedical representations of human sexuality were forged before the advent of evolutionary thought. According to an influential historiographical narrative founded by Thomas Laqueur (1986, 1992), homology thinking dominated biomedical views on human bodies from Greco-Roman antiquity to the late 17th century. Under the "one-sex model," male and female bodies were regarded as instantiations of the same type, while differing in degree of development, topological position, and relative value. In contrast, the economic, political, and cultural transformations of the 18th century led to a new articulation of the differences between the sexes. In the "two-sexes model," bodies started to be understood as essentially sexed, and sexual traits came to be clustered into two distinct natural kinds. Although this dichotomization affected all aspects of human anatomy, genitalia became the primary foundation of sexual dimorphism.

In the last few decades, historians of gender and sexuality have objected that Laqueur's two-stages narrative is built upon scarce and biased sources, and simplifies a much more nonlinear and complex history in which the themes of sameness and difference between the sexes cohabited in different time periods (Harvey, 2002; King, 2013; Linton, 2022; Stolberg, 2003).[1] Importantly for the case of the female

[1]The history of gynecology is a good trace of these progressive efforts for understanding sexual difference. Although the first uses of the word "gynecology" show up in the 18th century (McGrath, 2002), the emergence of gynecology as a medical discipline specifically addressed to study the female body has a long history (King, 2017). The progressive birth of gynecology had important consequences for the representation of sexual difference. While male bodies were the subject of the research on universals (reason, speech or posture), the study of female bodies was focused on their sexual and reproductive traits (Schiebinger, 2004). Nonetheless, the meaning attributed to female genital and reproductive organs throughout the 18th and 19th centuries captured a much larger span of disorders and pathologies than what is today captured by the discipline of gynecology. Before the emergence of the modern surgical discipline after 1850, a very wide variety of physiological and cognitive traits in women were attributed to their genital organs, based on the idea that women's reproductive functions dominated their entire being.

orgasm, Alison Moore (2018) has convincingly shown that two different versions of the homologous model cohabited well into the 19th century. In the Galenic tradition, which Laqueur takes as a reference framework, female genitalia were regarded as inverted versions of male genitalia, the testes being homologous to the ovaries and the penis to the vagina. However, the Hippocratic tradition considered the clitoris, not the vagina, as homologous to the penis. While both accounts regarded genital structures as anatomically homologous in the two sexes, their implications for the conceptualization of female pleasure were radically distinct: "in the Galenic view, women's pleasure is minimized and assumed to follow directly from coitus, while in the Hippocratic view women's pleasure is emphasized and located outside the zone of direct coital reception" (Moore, 2018, p. 55). As a consequence, the Hippocratic model permitted comparing sexual pleasure in men and women independently of their differing reproductive structures. In contemporary terms, the clitoris-penis homology allowed to separate *reproductive* homology from *erotic* homology.

Nonetheless, it is generally agreed that the Galenic and the Hippocratic traditions shared the belief that orgasm, or at least sexual arousal, had an analogous reproductive role in the two sexes. Generative substances were thought to be produced as a result of intercourse, and cause some kind of pleasant sensation when released in both men and women. The mammalian egg was only identified by Karl Ernst von Baer in 1827, and less than two decades later, the discovery of spontaneous ovulation led to the recognition of a radical separation between sexual pleasure and reproduction in women, as opposed to men (Laqueur, 1986). Up to then, the accumulated observations on induced ovulation and the use of the rabbit as a model organism in reproductive biology had only confirmed the assumption on a close link between pleasure and generation in females. In 1843, Theodor L. W. Bischoff reported the first unfertilized mammalian egg and found scars from ovulation in a dissected female dog that had not experienced any coitus. Bischoff's discovery was the first widely recognized evidence for spontaneous ovulation, and led to the conclusion that intercourse and ovulation were not necessarily linked. According to Laqueur, the discovery of spontaneous ovulation boosted the crisis of the homologous model as the primary way of representing the female body (1992, p. 213). Further research in reproductive biology, particularly in the cytological depictions of sperm and egg cells in mammals, might have contributed to what came to be perceived as fundamental differences between the anatomy and physiology of males and females. Thus, new embryological depictions of sperm and egg as active and passive, respectively, also influenced how new scientific narratives portrayed male and female pleasure as substantially different (McLaren, 2002, p. 337). Importantly, this new paradigm ceased momentarily with the search for a direct physiological mechanism connecting recreational and procreational sex in the female body. Nonetheless, the interest of physicians in women's pleasure did not disappear. Quite the contrary, medical writing on women's pleasure continued to be prevalent in the French context during the second half of the 19th century. Motivated by the belief that women had an equal potential for sexual pleasure, doctors continued to worry about women not having orgasms in marriage because they thought it would result in divorce and contribute to declining birth rates (see Cryle & Moore, 2011).

3 | FROM SEXUAL HOMOLOGY TO PHYLOGENETIC HOMOLOGY

With the rise of comparative anatomy and embryology in the 19th century, a new concept of homology strongly burst in the conceptualisation of sexual difference. In his celebrated conceptual taxonomy, Richard Owen (1843) distinguished between three kinds of correspondences of body parts, namely that between species (special homology), that between repeated elements within the body of an individual (serial homology), and that between a character of a species with that of the archetype (general homology). All these three homology relations had an influence on how sexual differences were represented and explained in pre-evolutionary comparative anatomy. Firstly, the correspondences between the sexes came to be conceptualized as an instance of homology, and more specifically as a subtype of serial homology. Secondly, sexual differences were homologized across species. Finally, sexual differences were conceptualized as resulting from the differentiation of a bisexual archetype represented by hermaphrodism. In the 1830s, the teratologist Isidore Geoffroy Saint-Hilaire defined hermaphrodism as "the coexistence in the same individual of both sexes or of some of their characteristics" (1836, p. 31). Geoffroy Saint-Hilaire relied on embryology to argue that the reproductive systems of males and females developed from a hermaphrodite stage, and that later in development, organs of one sex developed while those of the other sex remained rudimentary. The various types of hermaphrodism resulted from excess or defect of growth in the typical development of the reproductive organs of each sex (see Linton, 2022). The case of sexual differentiation instantiated a more general principle, namely the principle of compensation or the balancement law, according to which organs are enlarged at the expense of other organs that are rendered rudimentary (Appel, 1987, p. 76). Therefore, in the 19th century, comparative embryology allowed to understand sexual sameness from a comparative genealogical perspective. Sexual organs were homologous insofar as they "developed in a similar manner from a similar fundamental structure" (Watson, 1879, p. 52). The embryological definition of homology not only allowed the grounding of the identity relationships between external genitalia, but also between internal sexual organs. These included the testis and ovaries as derived from the genital ridge, the spermatozoa and ova from the primordial germ cells, the Sertoli and granulosa cells from the sex cords, and the male and female derivatives of the mesonephric tubules, ducts and ligaments, and the paramesonephric ducts. In the context of the theory of parallelism, remnants of sexual organs, such as incomplete uterus or vaginas in males, were identified in "lower animals", as well as in human pathologies (Watson, 1879).

After the publication of the *Origins of species*, the transformation of homology into a historical or phylogenetic concept led to

interpreting the previously established anatomical and embryological correspondences between the sexes as an instance of common ancestry. In the reedition of *Elements of comparative anatomy*, Carl Gegenbaur reinterpreted hermaphrodism as the ancestral stage preceding the evolution of sexual differentiation due to relative changes in the size of sexual organs. According to Gegenbaur, sexual differentiation not only affected generative substances and reproductive organs, but entailed an integral differentiation of individuals: "The separation of the sexes affects the whole of the organism, for it produces a series of changes in each sex, which affect organs that had primitively little to do with the sexual function. Sexual differentiation is completed when the two kinds of organs are given over to different individuals. Thenceforward for reproduction, not only two different substances, semen and ova, and two different organs for producing them, are necessary, but also two individuals; these are distinguished as male and female" (Gegenbaur, 1878, p. 54).

In *The Descent of Man*, Darwin credited Gegenbaur's hypothesis of the hermaphrodite ancestor of vertebrates, although he also speculated that rudimentary sexual traits in mammals might have resulted from hereditary correlations between the sexes: "to account for male mammals possessing rudiments of the accessory female organs, and for female mammals possessing rudiments of the masculine organs, we need not suppose that their early progenitors were still androgynous after they had assumed their chief mammalian characters. It is quite possible that as the one sex gradually acquired the accessory organs proper to it, some of the successive steps or modifications were transmitted to the opposite sex" (Darwin, 1871, p. 208). According to Ghiselin, these early speculations on the hermaphrodite ancestor reflect a conflation between sexual and phylogenetic homology, and Darwin's hesitation between phylogenetic and developmental explanations of sexual homology illustrate the incipient historical decoupling between these two homology concepts (Ghiselin, 1969, pp. 118–119, and Ghiselin, 2005).[2]

The concept of embryological homology also joined the discussion on the evolutionary origins of sexual differentiation. Haeckel's biogenetic law was the core conceptual framework to connect the development and evolution of sexuality. In this new context, embryonal sexual differentiation was believed to reflect the phylogenetic history of mammals, and some sexual characters were interpreted as vestigial traces of the evolutionary past. "Atrophied" sexual organs, that is, organs reduced in size as compared to their homologous counterparts, such as male breasts as compared to females, or glans clitorises as compared to penis, were reinterpreted as vestigial characters. On many occasions, the development and evolution of sexual characters were regarded as progressive, teleological processes. Evolutionary teleological explanations of human sexuality were embedded in the wider historical teleological theory of sexuality typical of the 19th century, first formulated in humanistic fields outside evolutionary biology (Moore, 2021).[3] The early 20th-century debates on the anatomical locus of female pleasure in the field of sexology were highly influenced by this historical interpretation of the *telos* of human sexuality. These included, among others, Havelock Ellis' ideas on bisexuality, Gregorio Marañón's reflections on gender, and Magnus Hirschfeld's concept of primeval inter-sexuality (Bauer, 2012; Moore, 2015). In the following section, we concentrate on the interaction between psychoanalysis and evolutionary perspectives on human sex. We will contrast this approach with the later quantitative studies of human sexuality, which were influenced by a different, quantitative school in evolutionary biology, and set up the context for contemporary evolutionary debates on the female orgasm.

4 | SEXUAL HOMOLOGY AND THE MOVING LOCUS OF FEMALE PLEASURE

While anatomical homologies between genitalia were well-established, differing views about the anatomical locus of female sexual pleasure continued circulating throughout the 19th century, and it was not until the early 20th that the questioning of clitoral orgasm became mainstream in medical practice. Socio-political imperatives on the distinction between men and women, together with new biomedical views of sexuality were involved in this transition (Moore, 2018). These included dismissive medical views of masturbation, but the psychoanalytic theory of the vaginal orgasm was undoubtedly the most influential scientific theory in this regard. In his *Three essays on the theory of sexuality*, Freud (1905/2017) argued that, while men remain consistent in retaining their penis as the core anatomical locus of sexual pleasure, women experience in their transition to maturity a "transfer" of their center of sexual sensitivity from the clitoris to the vagina.

Freud's transfer theory reconciled two modern views on sex, namely the theory of bisexuality, and the distinction between males and females based on their complementary roles in reproduction (Freud, 1905/2017, p. 142). At various places in his works, Freud notes that sciences do not provide a clear-cut distinction between the sexes, and interprets this anatomical fuzziness as an indication of a shared, primary bisexuality: "(science) draws your attention to the fact that portions of the male sexual apparatus also appear in women's bodies, though in an atrophied state, and vice versa in the alternative case. It regards their occurrence as indications of bisexuality, as though an individual is not a man or a woman but always both-merely a certain amount more the one than the other" (Freud, 1964, p. 114).

The themes of bisexuality, hermaphrodism, and androgyny were ubiquitous within and outside the scientific circles of Fin-de-siècle Vienna (McEwen, 2012). In his training as a biologist, Freud became influenced by the theory of bisexuality from multiple perspectives that

[2]As we will see later, contemporary discussions show that the distinctiveness between developmental and historical homology concepts, as applied to serial characters, is not so neat as Ghiselin expected (Fusco, 2022).

[3]As an illustration, sexual promiscuity was regarded as the primitive state in human history, followed by a later control of sexuality, and sexual pathologies were read as atavisms (Moore, 2021).

deeply influenced his conception of human sexuality (Freud, 1905/2017, p. 142). These included the work of the physiologist Wilhelm Fliess on the "bisexual constitution" of every living organism (Heller, 1981), the comparative studies on hermaphroditism by the zoologist Carl Claus (Sulloway, 1992), and Darwin's theory on the original bisexuality of humans (Bauer, 2012). Under the Darwinian perspective, bisexuality (understood as the coexistence of male and female traits in the same individual) corresponded to an earlier, undifferentiated stage of development and evolution, while the progressive differentiation between the sexes was the result of natural selection (see Angelides, 2001; ch. 2).

The influence of historical explanations of sexuality can be found in Freud's reliance on teleological notions on how underlying homologies gradually diverge throughout evolution (Moore, 2021): genital homologies "lead us to suppose that an original bisexual disposition has, in the course of evolution, become modified into a unisexual one, leaving behind only a few traces of the sex that has become atrophied" (Freud, 1905/2017, pp. 243–244). A later evolutionary legitimation of the transfer theory with an important impact on psychoanalysis was proposed by the ethologist Frank A. Beach in the 1940s. According to this theory, the vaginal orgasm was a recent evolutionary acquisition of the human species, linked to the evolution of higher intellectual abilities related to the self-control of sexuality (Beach, 1948). This explained that only a few women were able to experience orgasm, and transformed the vaginal orgasm into an "evolutionary ideal" (Sherfey, 1966).

Freud's transfer theory departed from the widely held recognition of embryological, anatomical, and phylogenetic homologies between the genitals of the two sexes, but required the fragmentation of female pleasure into two distinct erogenic zones. While the clitoris was seen as a male part in women's bodies, the vagina constituted a distinctive female part without a male homologous correlate. This anatomical differentiation within the female genital system had no precedent in previous theories on the anatomical basis of female pleasure (Tuana, 2004). Physiological homology, in Freud's eyes, was linked to psychological homology, insofar as each genital zone was associated to the distinct sexual behaviors attributed to the two genders (Traub, 2001). Therefore, the transition of libido from the clitoris to the vagina permitted to overcome anatomical homology and achieve a psycho-physiological differentiation. As many commentators have noted, this postulated transition was not a descriptive, but a normative one "attempting to reconcile women's physiology with a heterosexual imperative" (Traub, 2001, p. 153). At the turn of the 20th century, sexuality constituted in Vienna the primary idiom through which topics and anxieties related to modern society were reflected (Luft, 2003), and Freud's theory can only be understood within this cultural context. Thus, according to the transfer theory, the proper form of femininity was achieved by restraining sex to its reproductive function, and the lack of vaginal orgasms during intercourse was theorized as the failure of culture to resignify women's bodies into their appropriate societal roles (see Koedt, 1970; Laqueur, 1986; Moore, 2018).

The Freudian transfer theory constituted the basis for dominant narratives of female sexuality in the following decades, portraying women as either lacking sexual passions or as victims of pathological affections (Maines, 2001). After the influence of Freudian ideas in the post-war, North American context, frigidity was redefined and diagnosed as a lack of orgasm in penetration (Cryle & Moore, 2011). In such a context, a new generation of sexologists stood up for the modern expectation of an egalitarian depiction of sexual pleasure, and resorted to the concept of sexual homology as a reaction against the psychoanalytic theory (Gerhard, 2000; Moore, 2018). Nonetheless, Freud recognized his limitations as a man in theorizing on female pleasure and hoped his female disciples would elaborate further on the topic. The resulting tension is illustrated in the complex figure of Marie Bonaparte (1949). On the one hand, Bonaparte wanted to embrace the androgyny that psychoanalysts read on clitoral pleasure, interpreting it as a positive sign of the gender equality brought about by modern civilization. On the other hand, she assumed the Freudian dichotomization of erogenous zones in the female body, and applied its normative implications to her own. By surgically relocating her clitoris, she attempted to conciliate masculine and female pleasure (Cryle & Moore, 2011, pp. 222–247).

In the early 1950s, Alfred Kinsey and his group at the Institute for Sex Research distanced themselves from the psychoanalytic approach to sexuality, and embraced an empirical, statistical approach that prioritized quantitative data over subjective and individual case studies. Kinsey decided to study human sexual behavior after a failed career as an evolutionary taxonomist. Although this meant focusing on the proximate causes of sexual behavior, he kept in touch with debates precipitating into the Modern Synthesis, and applied the methods for data gathering and ordering, as well as the processual approaches learned from evolutionary biology, to human sex research (Drucker, 2014). In a groundbreaking treatise, Kinsey and collaborators defended the clitoral basis of the female orgasm by relying again on the embryological homology between male and female genitalia: "In the female and male mammal the external reproductive organs, the genitalia, develop embryologically from a common pattern. They are, therefore, homologous structures in the technical meaning of the term" (Kinsey et al., 1953/1998, p. 571). In their view, the comparison of female and male sexual behavior depended on a "better understanding of the anatomy and physiology of sexual response and orgasm" (p. 575). After five chapters devoted to comparing the anatomy and physiology of human sexuality in both sexes, they concluded that there was no clear way to classify men and women into two different sexual groups in terms of orgasmic capacity, sexual behavior, or body composition: there was one single orgasm in females, and it was homologous to the male orgasm.

A decade later, William Masters and Virginia Johnson continued this turn toward an empirical approach to human sexuality, publishing the results of their physiological experiments in their 1966 book *Human Sexual Response*. Reporting on Kinsey and colleagues' research, Masters and Johnson argued for the role of the clitoris in the female orgasm on the basis of anatomical and physiological homologies between male and female sexual response. Masters and Johnson considered the psychological and physiological dimensions

as integrated aspects of the female orgasm: "For the human female, orgasm is a psychophysiologic experience occurring within, and made meaningful by, a context of psychosocial influence. Physiologically, it is a brief episode of physical release from the vasocongestive and myotonic increment developed in response to sexual stimuli. Psychologically, it is a subjective perception of a peak of physical reaction to sexual stimuli" (Masters and Johnson, 1966, p. 127). However, they developed methods to describe and measure the physiological and psychosocial dimensions of the human orgasm independently. Along with the subjective communication of sensory experience, ways of measuring the physiological processes described were needed for turning orgasm into an objective and comparable trait. Their major contribution in this regard was the proposal of a "human sexual response cycle" comprising four phases of sexual response—excitement, plateau, orgasm and resolution. Assuming that the sequence of physiological changes was the same in both sexes, Masters and Johnson identified and measured those neurological, muscular, and vascular parameters that were comparable in men and women, concluding that male and female orgasms were homologous in terms of duration, intensity, and underlying mechanisms.

In concluding that the clitoris was the main erogenic zone in the female body, human sex research from the 1950s and 1960s had a great impact on depathologizing female pleasure in biomedical studies and treatments of human sexuality. In addition, between the late 1960s and the mid-1970s, there was an explosion of feminist analyses of the political meaning of sexuality that critiziced the heteronormative biases of psychoanalysis and relied on the new "facts of biology" to reclaim women's sexual autonomy (Gerhard, 2000).[4]

From a comparative scope, sex studies of the female orgasm had a revolutionary impact as well. The new sexologists tended to assume that the female orgasm was specific to humans and therefore did not discuss it in an evolutionary context. Nonetheless, their focus on anatomy and function, and the description of orgasm as a sequence of physiological events, set the basis for objective definitions of sexual response, allowing for the study of the female orgasm as a natural trait that could be found outside the human species (Musser, 2012). As a matter of fact, although Kinsey and colleagues concluded that "orgasm is infrequent and possibly absent among females of most species of mammals" (Kinsey et al., 1953/1998, p. 135), they did refer to endocrinology reports of female orgasms in other species,[5] and admitted that the main obstacle for testing its existence was a lack of adequate criteria for its identification and interpretation outside humans (pp. 628–629).

[4]This included some exceptional psychoanalysts (Sherfey, 1966), but the most influential essay was "The Myth of the Vaginal orgasm," published in 1970 by Anne Koedt (1970) in a radical-feminist journal. Citing Masters and Johnson, Koedt blamed Freud's "invention" of the vaginal orgasm and the devastating psychological consequences that diagnoses of frigidity had for women. In turn, Koedt's essay inaugurated a further historiographical myth in post-1970s feminist vindications of the clitoral orgasm, according to which the clitoral orgasm had been broadly repressed in the late 19th-early 20th century.

[5]In the 1920s and 1930s, endocrinologists discussed orgasm in rabbits in connection to induced ovulation (Marshall & Verney, 1936; Walton & Hammond, 1928), and sperm transport (Parker, 1931).

Between the late 1960s and early 1970s, primatologists and anthropologists such as Suzanne Chevalier-Skolnikoff started to study behavioral signs indicating orgasmic activity in female primates (see Musser, 2012). In turn, comparative studies of sexual response prepared the terrain for an evolutionary approach to the female orgasm. On the one hand, studies of sexual behavior in primates opened the door for evolutionary interrogations on the adaptive role of the female orgasm. On the other hand, the critics of adaptationism started challenging selectionist explanations of the female orgasm, bringing homology back to evolutionary explanations of female sexuality.

5 | THE FEMALE ORGASM AS AN EVOLUTIONARY ADAPTATION

In the early 20th century, the fall of evolutionary morphology and the acceptance of natural selection as the only guiding force of evolution, led to a proliferation of adaptive approaches to human sexuality that pushed homology thinking to the background. In the Darwinian view of evolution, homologous parts differed through adaptation, not only with the external and internal environment, but also with parts of the other sex. In this new explanatory context, the theory of sexual selection became the core evolutionary force accounting for sexual differentiation and had major implications for the representation of female sexuality. Placing female preferences and choice at the explanatory center challenged the passionless Victorian depiction of female sexuality (Thornhill & Gangestad, 2008, p. 3), but also reinforced the myth of the monogamous, "coy" female courted by undiscriminating males. Female promiscuity only started to be considered in the late 1970s, when the female perspective was introduced in evolutionary discussions on human sexuality (Hrdy, 1981/2009). For instance, genetic hypotheses for polyandry alleged that the extended sexual receptivity of female primates, as provided by their clitoral ability to experience orgasms, was a mechanism selected to generate genetic variation through mating with multiple males. However, under the assumption that reproductive functions should explain the evolution of female sexuality, the female orgasm became an evolutionary mystery (Buss, 2016), or "an adaptive paradox" (Kennedy & Pavličev, 2018). The physiological complexity of the female orgasm made the reasons for the evolutionary maintenance of this character even more mysterious, given that nonfunctional traits are expected to deteriorate unless they are under selection.

To solve this paradox, the earliest adaptive hypotheses attempted to unravel a direct role of the female orgasm in reproduction. In *The naked ape*, Desmond Morris speculated that the exhausting satisfaction following orgasm in women had the effect of keeping their bodies horizontal and retaining the seminal fluid (Morris, 1967, p. 79). Later in the 1990s, evolutionary biologists recovered a physiological hypothesis dating back to 1854 that postulated a link between female orgasm and sperm transport (see Levin, 2011a,b). After the discovery of spontaneous ovulation, the

role of uterine contractions in assisting sperm transport replaced induced-ovulation as the main mechanism for reuniting orgasm and conception in the female body. The new evolutionary versions of the upsuck hypothesis postulated that uterine contractions released by climax were functional in retaining the sperm inside the reproductive tract, thus promoting sperm competition (Baker & Bellis, 1993; Thornhill et al., 1995). The upsuck hypothesis was popularized by many authors in the 2000s (see Lloyd, 2005, pp. 216–217), but was widely discredited in the following decades. Despite some recent attempts to restore it (King et al., 2016), current evidence suggests that the female orgasm plays no role in sperm transport (Levin, 2011a), nor is there any correlation between female orgasms and offspring number (Zietsch & Santtila, 2011).

Alternative adaptive hypotheses have attempted to find an indirect role of orgasm in improving female reproductive success. Most rely on pair bonding, considering orgasm as an adaptation that motivates females to engage in intercourse outside the fertile phase of the cycle, and creates long-term relationships with their male mates. First introduced by Morris (1967), different versions of the pair-bonding hypothesis were formulated during the 1970s (see references in Lloyd, 2005, pp. 44–77). Nonetheless, the fact that vaginal intercourse alone is not the most reliable way to achieve orgasm in female primates, led advocates of pair-bonding theories to emphasize mate selection (Alcock, 1978, 1980; Nebl & Gordon, 2022; Prum, 2017). Females would select mates arousing them to orgasm during intercourse, either because they just like it (Prum, 2017) or because they indirectly select for prosocial empathy (Kennedy & Pavličev, 2018).

In her influential book *The Case of the Female Orgasm*, Elisabeth Lloyd (2005) undergoes an exhaustive critical review of adaptive explanations of the female orgasm, outlining the theoretical and social biases shaping biological research. The core, general argument against adaptive hypotheses is their lack of solid evidence. Ultimately, selectionist theories on the female orgasm fail to meet standards of scientific corroboration, being an instance of untestable narrative explanations (Gould & Lewontin, 1979). The case of the female orgasm also illustrates how research paradigms lower the standards of good science whenever they meet the predictions of the theory they aim to corroborate, and preclude research in other directions. Thus, the statistical shortcomings of studies supporting the upsuck hypothesis passed widely unnoticed because they met the adaptationist expectation of a connection between female orgasm and reproduction, and adaptationist hypotheses were not even contrasted with alternative ones such as the byproduct hypothesis (see below). Secondly, Lloyd outlines the effects of the ancient, persistent social trend of considering female sexuality as inevitably tied to procreation. Adaptive theories look at the female orgasm during intercourse, and hence only regard it as a reproductive trait, rather than as just sexual behavior. Instead, Lloyd suggests considering sexuality as an independent set of activities "which are only *partially* explained in terms of reproductive functions" (Lloyd, 1993, 140). Examples of nonreproductive sexual behaviors include female same-sex sexual behavior, or copulation outside the fertile phase of the ovarian cycle in mammals and birds (Thornhill & Gangestad, 2008).[6]

Evolutionary debates on the adaptive role of the female orgasm make scarce references to the anatomy, physiology, development, and phylogeny of female sexuality, the homology concept being virtually absent from the discussion. This style of reasoning reflects a general trend in adaptationist thinking. Insofar as the goal of selection-based explanations is to understand why traits are preserved, the anatomy and physiology of sexual traits tend to be seen as irrelevant. The focus on selective pressures, together with the consideration of the female orgasm as a uniquely human trait, erased the comparative and phylogenetic dimensions of female sexual pleasure from evolutionary debates. Since the 1980s, the critique of adaptationism and the return of homology thinking has impacted evolutionary studies of human sexuality, and new hypotheses on the origin and evolution of the female orgasm have come to the fore.

6 | BACK TO SEXUAL HOMOLOGY: THE BYPRODUCT HYPOTHESIS

In the late 1970s, the crisis of the adaptationist program led to a renaissance of the interest in homology in evolutionary biology that rapidly entered the debate over the female orgasm. Surprisingly enough, the first explanations of the female climax as a side-effect of sexual homology (what later will be known as the byproduct hypothesis) can be found in the seminal works of the adaptationist approach to human sexuality. In *The naked ape*, Desmond Morris refers to the homology between clitoris and penis to speculate that the female orgasm might be, in origin, a "borrowed male pattern" (Morris, 1967, p. 80) that was later co-opted for a different function. A decade later, Donald Symons advanced the idea of the female orgasm as a side-effect of selection on male orgasm that was not in need of an independent adaptive explanation: "The female orgasm may be a byproduct of mammalian bisexual potential: orgasm may be possible for female mammals because it is adaptive for males" (Symons, 1979, p. 92). However, Symon's version of the byproduct hypothesis still entails an ambiguous concept of bisexuality, and seems to refer to a psychological or a behavioral concept of orgasm, rather than to any precise developmental, anatomical, or physiological notion of sexual homology (see Lee, 2013). The description of the female orgasm as the behavioral homolog of male ejaculation appears as well in several reports on macaques from the 1970s and 1980s (see references in West-Eberhard, 2003; pp. 276–277). Nonetheless, the articulation of a developmental hypothesis on the evolution of the female orgasm seems to have required the reconceptualisation of

[6]It has been argued that the conflation between orgasm and conception has led to important misconceptions on the male side as well. Orgasm and ejaculation are also different physiological phenomena in males, and only seminal emission, not orgasm itself, is essential to conception. Therefore, "there is no better adaptational explanation for the existence of men's orgasm than for the existence of women's" (Komisaruk, 2006, p. 12).

homology in developmental terms that took place in the 1980s. In the frame of the nascent evolutionary developmental biology, or evo-devo, two traits are homologous if they share a common developmental cause that explains their identity relation (Roth, 1984; Wagner, 1989). Relying on his previous criticisms of adaptationism and his advocacy for developmental and historical constraints, it was Stephen Jay Gould who provided the first evolutionary developmental version of the byproduct hypothesis. In invoking the female orgasm to advocate for nonadaptive explanations as legitimate explanatory alternatives, Gould (1987a) applied the classical notion of embryological homology to articulate an evolutionary explanation of the female orgasm. In Gould's view, the two sexes are "variants upon a single ground plan, elaborated in later embryology". As a consequence, sexual differences do not need to be independently explained by adaptive criteria: just like "[m]ale mammals have nipples because females need them" (Gould, 1993, p. 83), females have a clitoris because males need their penis and the ejaculation associated to orgasm to reproduce. Ultimately, the evolutionary origin and maintenance of the female orgasm is a consequence of the selection on the male orgasm, and the associated constraints entailed by the common development of genitalia. In her 2005 book, Lloyd favored the byproduct hypothesis on the basis of the lack of evidence for adaptive explanations, and later contributed herself with new empirical evidence in support of the byproduct hypothesis. Together with Kim Wallen, Lloyd argued that higher variability in clitoral length from the shaft to the clitoral glans tip, as compared to the penis, suggests a lack of selective pressures (Wallen & Lloyd, 2008; but see Lynch, 2008).

The byproduct hypothesis is not free of problems, and constitutes a current matter of controversy. Objections can be grouped into two major categories, both related to the criteria and implications of relying on sexual homology to explain the evolution of the female orgasm. Firstly, genetic, anatomical, and physiological specificities of female sexual response cast doubts on the argument that the female orgasm can be exclusively explained as a byproduct of male physiology. These include the evolutionary preserved complexity and intensity of the female orgasm despite the lack of a function, the involvement of the pituitary in the female, but not in the male, orgasm (Huynh et al., 2013), and the lack of a clear sex-genetic correlation for this trait, which suggests that different genetic factors underlie male and female orgasmic function and variance (Zietsch & Santtila, 2012; but see Wallen et al., 2012). Secondly, the existence of sexual homology does not preclude that of independent, selective pressures on the female orgasm. In her book *The Woman That Never Evolved*, Sarah Hrdy (1981/1999) claimed that Symons' byproduct explanation dismissed female sexuality in this regard. Despite their anatomical homology, sexual organs have been subject to different selective pressures that are neglected by the critics of adaptationism: "we cannot explain special features of the clitoris such as its size, positioning, or degree of enervation merely by examining selection pressures on males to have a penis designed in a particular way. Once again, it was an error for evolutionists to assume glibly that by examining one sex we could learn all we needed to know about the other" (Hrdy, 1981/1999, p. 251). Gould's (1987) article also sparked a heated scientific exchange when John Alcock objected in a letter to the editor of *Natural History* (1978) that the clitoris and the female orgasm should not be conceptualized as a lesser, vestigial version of male anatomy and pleasure. In the Hrdy (1981/1999) bibliographical update of her book, Hrdy includes Gould's paper and criticizes him for the same reason: the byproduct hypothesis endorses and reinforces the assumption that female sexuality is a derived consequence of male sexuality, ultimately echoing the old view of sexuality being originally, and therefore fundamentally, masculine. In her reply to feminist critiques of the byproduct hypothesis, Lloyd warns about the perils of conflating biological function with social value: arguing for or against the hypothesis that the female orgasm is an adaptation does not say anything about the value of the trait (Lloyd, 2005, pp. 139–143).

Even if the notion of sexual homology grounding the byproduct hypothesis does not exclude a phylogenetic perspective, the reference to developmental correlations between the sexes does not allow itself to situate the female orgasm in a historical framework. In the last years, evo-devo studies of female sexuality have gone beyond the concept of sexual homology to cover precisely this gap, and trace the phylogeny of the female orgasm. As we will see in the next section, the incorporation of new homology concepts has been instrumental in this new move permitting to connect the physiology of human pleasure with that of other species.

7 | BACK TO PHYLOGENETIC HOMOLOGY: THE OVULATORY-HOMOLOG HYPOTHESIS

Evolutionary explanations have tended to implicitly or explicitly consider the female orgasm as a uniquely human (or, at most, primate) trait. In a series of recent papers, Mihaela Pavličev and Günter Wagner have revised this assumption and revisited the evolutionary enigma of the female orgasm from a comparative scope (Pavličev & Wagner, 2016; Wagner & Pavličev, 2017; Pavličev et al., 2019). Pavličev and Wagner follow Lloyd's critique of adaptationism and bet for "homology thinking" as an alternative explanatory approach to the evolution of the female orgasm (Wagner & Pavličev, 2017, pp. 1–3). However, differently to Lloyd, they argue that extant theories (including both adaptive and byproduct theories) have been so far focused on the human orgasm, and remained incapable of tracing back the evolutionary origin of the trait. Instead, the elucidation of the origination of the female orgasm requires a *phylogenetic* notion of homology based on a comparison of this character across lineages. Under this premise, Pavličev and Wagner advocate for an alternative explanation of why the female orgasm has no evident reproductive functions in humans. According to their "ovulatory-homolog" hypothesis, the female orgasm and the ovulation process were physiologically linked in an ancestral stage of mammalian phylogeny, but became later decoupled in eutherians.

In contrast to the well-established anatomical and developmental data supporting sexual homologies, the postulation of a phylogenetic

homology for the mammalian female orgasm required the collection of new experimental and phylogenetic evidence. In a recent experiment, Pavličev and collaborators showed that copulation-induced ovulation in rabbits was affected by the administration of fluoxetine, a well-known drug (the famous "Prozac) that inhibits orgasm in humans (Pavličev et al., 2019). Although these results do not rule out other explanations, such as a convergent mechanism being responsible for the response to fluoxetine, they provide strong support for the hypothesis that the female primate orgasm derives and still shares some mechanistic basis with the neuroendocrine reflex inducing ovulation in other mammals. Moreover, this experimental approach to homology represents a substantial step in testing homology-based explanations of the female orgasm. From a phylogenetic perspective, the ovulatory-homolog hypothesis is based on phylogenetic data suggesting that male-induced ovulation is the ancestral condition in mammals, while spontaneous ovulation is a derived mode of the ovarian cycle originating later in several eutherian clades. More recently, Pavličev and collaborators have strengthened the phylogenetic support of the ovulatory-homolog hypothesis with a comparative anatomy of the development of male and female external genitalia in different mammalian species (Pavličev et al., 2022). While after early joint development, male genital and urinary tracts always integrate into the phallus, in females there is a lot of structural and positional interspecific variation. In species with spontaneous ovulation, the clitoral glans tends to be far apart from the vagina and is not functionally linked to ovulation, while in species with copulation-induced ovulation, the clitoral glans is generally located inside the vagina.

The individuation of the female orgasm as a comparable, mammalian trait, entails establishing homological relationships at different levels. Concerning morphological homology, the anatomy of the female orgasm has a major role in the postulation of the ovulatory homolog hypothesis. The comparative anatomy of female genitalia is an understudied topic that was hardly ever mentioned in connection to the evolution of the female orgasm until Pavličev and Wagner's research. The intermittent neglect of the clitoris has been widely documented in the history of human anatomy, and is still patent in recurrent omissions of the organ from contemporary anatomical drawings (Moore, 2018; Tuana, 2004). Recent studies describing the external and internal anatomy of the clitoris, including not only the clitoral glans, but also the paired bulbs and corpora, characterize their results as rediscoveries of forgotten anatomical works (O'Connell et al., 2005). This new research has raised interest among medical humanities and social science scholars, who have reflected on the consequences of considering the holistic nature of female pleasure for the debate on the locus of female orgasm (Blechner, 2017; Moore, 2018; Tuana, 2004). In the evolutionary terrain, a parallel debate on the role of clitoral anatomy has been raised in the reactions triggered by the ovulatory homolog hypothesis. Komisaruk (2016) pointed out that Pavličev and Wagner (2016) had not been careful enough in distinguishing between the external glans and the internal corpus of the clitoris, which can also be stimulated in copulation through the vaginal wall. In their reply, Wagner and Pavličev (2016) acknowledge that more anatomical knowledge of the evolution of clitoral anatomy is needed, but argue that the externalization of the clitoral glans from the vagina, as associated to the evolution of spontaneous ovulation, gives a robust anatomical support for their hypothesis.

Although a good deal of the debate on the female orgasm has concerned the anatomical locus of sexual stimulation, orgasm itself is not a morphological character. Rather, postulating the homology of the female orgasm among mammals involves identifying sameness at a physiological level. This entails a major difficulty in applying the homology concept, which has classically been used for morphological characters, to the female orgasm. Nonetheless, in the last years, various voices have argued that there is no need to restrict the concepts of homology and novelty to structural components. Activities (Love, 2007) or bodily functions (Brigandt, 2017) can be equally homologized. In defining orgasm as a neuroendocrine reflex triggered by clitoral stimulation, the homology of the mammalian orgasm illustrates this notion of complex bodily parts as composed of both structures and functions open to evolutionary modification (Brigandt, 2017). In this regard, the ovulatory homolog hypothesis moves the definition of the human female orgasm from a subjective psychophysiological experience to that of an objective, comparable trait. As mentioned above, the founders of modern sexology provided the first definitions of the human orgasm in terms of physiological homology. But in focusing on humans, they framed orgasm as an integrated step of a whole sexual response continuum, and therefore did not exclude the subjective dimension of this process. The individuation of the female orgasm as a neuroendocrine reflex makes it possible to abstract away the subjective dimension of the female orgasm, insofar as a subunit of the same process can be identified as homologous. This partitioning of the sexual response reflects the factorial or combinatorial nature of homology, and, therefore, the fact that homological relationships can be partial (Fusco, 2022).

The definition of female orgasm as a neuroendocrine reflex has been one of the most controversial issues in the scientific reception of the ovulatory homolog hypothesis, as shown in a recent article in *Scientific American* covering the reactions of two neuroendocrinologists to the experimental results on rabbits (Lewis, 2019). Julie Bakker, from the University of Liège, pointed out the limitations of studying orgasm in animal models: "There's no such thing as orgasm in rabbits"; "it is more like a light switch, in which male stimulation triggers the brain, which triggers ovulation". In the same line, Raúl Paredes, from the National Autonomous University of Mexico, blames as reductionistic the definition of orgasm as an induced reflex: "This is a human construct because, aside from the physiological changes that can occur during sex, the definition involves feelings of pleasure" that "can't be measured in animals". In a response paper, Wagner and Pavličev (2017) argue that, in relying on subjective experiences and peripheral signs of excitement, purported definitions are rather descriptions, of the female orgasm. Defining orgasm as a neuroendocrine reflex they argue is the only way to provide an objective, comparable definition allowing us to trace back its evolutionary origin.

The search for the evolutionary delimitation of the female orgasm not only shows how definitions of a character change substantially under different notions of homology. It illustrates as well the interdependency between the homology and the novelty concepts. Is the female primate orgasm an evolutionary novelty with no evolutionary precedent in the mammalian lineage, or rather a character state derived from the ovulatory reflex in ancestral species with coitus-induced ovulation? (Wagner & Pavličev, 2017). Depending on whether the human female orgasm is individuated as a novelty or as a derived character, its phylogenetic origin might be traced back to primates, to mammals with spontaneous ovulation, to the ancestor of mammals, or even to the origin of amniotes.

Following the reasoning that the female orgasm was ancestrally connected to ovulation, a recent paper suggests that this trait could indeed be traced back to the reflex of ovulation in the transition from external to internal fertilization (Lodé, 2020). Most evolutionary biologists would likely identify the phylogenetic origin of the male orgasm in the origination of amniotes, when the developmental structure for both the penis and the clitoris originally evolved, together with internal fertilization (Sanger et al., 2015). Hence, the assumption that the female orgasm originated in mammals, the male orgasm being much more ancestral, might be revised. If the reflexes of ovulation and ejaculation are proved to share evolutionary roots, one might end by harmonizing phylogenetic and sexual homology in a unitary explanation for the evolution of orgasm. Less ambitiously, the ovulatory homolog hypothesis assumes that orgasm is at least present in mammals. Therefore, the female primate orgasm seems to be understood as a character state of a single, homologous trait that has undergone evolutionary modifications, including a disentanglement between female orgasm and ovulation in eutherians. After this evolutionary autonomisation from ovulation, the female orgasm would qualify as an evolutionary innovation. Nonetheless, the externalization of the clitoral glans and the evolution of spontaneous ovulation have independently evolved multiple times in mammals (Pavličev & Wagner, 2016). Accounting for the origination of these homoplastic traits might therefore require additional explanations. In particular, one might argue that the specificity of female sexuality in primates is not only determined by the decoupling between orgasm and reproduction, but rather by psychosocial factors. In primates, female sexuality is more influenced by cognitive and relational aspects, and extended female sexuality, as connected to the evolution of hidden ovulation, reaches a unique scope in humans (Thornhill & Gangestad, 2008).

It is generally assumed that selection-based and homology-based explanations constitute different explanatory agendas addressed to solve nonoverlapping problems in evolutionary biology (Amundson, 2005). Just like adaptive hypotheses do not aim at accounting for the origination of the female orgasm, the individuation of the female orgasm as the neuroendocrine mechanism decoupled from induced ovulation does not provide the adaptive causes for its evolutionary persistence. Instead, the aim of homology-based explanations is to shed light on the origination of the female orgasm and individuate this trait as a historical unit. Indeed, an explicit conceptual distinction motivating the ovulatory homolog hypothesis is that between evolutionary explanations of the origin of a trait, and those accounting for the maintenance of this trait. From this perspective, extant hypotheses on the evolution of the female orgasm might not be incompatible, but rather refer to different stages of the evolutionary history of the trait. Thus, the decoupling between orgasm and conception would account for the origination of the female orgasm, the byproduct hypothesis might provide the developmental mechanisms for the maintenance of correspondences between the anatomy and physiology of pleasure in the two sexes (see Davis, 2019), and adaptive explanations would unravel the selective forces behind its evolutionary persistence. Nonetheless, mechanistic approaches to character evolution can also improve adaptive explanations, and even open new terrains for adaptive hypotheses. For instance, several studies have documented a prevalence of sexual symptoms during menopause in humans, including poor arousal and orgasm (e.g., Nappi & Lachowsky, 2009). This association might be interpreted as supporting the hypothesis on the ancestral connection between orgasm and ovulation, but the tenacity of orgasm, namely the fact that it does not disapear with the cessation of ovulation despite a decrease in intensity, might also indicate that orgasm has other functions not related to reproduction, or support the non-function claims of the byproduct hypothesis. At this point, it is important to emphasize that physiological and evolutionary functions are not synonymous. The evolutionary function of the uterus is obviously related to reproduction, but females do not lose their uterus once they reach menopause and lose their physiological ability to reproduce. Many traits are only functional during some stages of the life cycle, and are evolutionarily preserved for that reason. But what matters for our argument is that the postulation of an ancient evolutionary association between ovulation and female orgasm opens the way for testing new mechanistic hypotheses on, for instance, the physiological connection between menstruation and orgasm. Moreover, the hypothesis on the independent evolution of the female orgasm requires unraveling the evolutionary forces behind the evolution of spontaneous ovulation in mammals and the associated changes in genital anatomy.

8 | CONCLUSIONS

The female orgasm is one of the most contested topics on human sexuality, charged with speculation, storytelling, and gender biases. At this crossroads, shifts in the reference of comparison and the application of different definitions of homology to the understanding of sexual anatomy and pleasure have been the underlying conceptual foundation for theories on female sexuality. Sexual homology was the prevalent model for understanding the physiology of female pleasure until the mid-19th century, when the rupture of the link between sexual pleasure and reproduction became the main foundation for the distinctiveness of sexuality in women. At the turn of the new century, two different disciplines concerned with the study of sexuality, namely psychoanalysis and evolutionary biology, explored different strategies to relink sexuality and reproduction in the female body. This reinstated link reframed the contrast between males and females under a new model of sexual complementarity, where homological relationships were downgraded and the two sexes played the role of matching pieces in the puzzle of reproduction. The

concept of sexual homology recovered its centrality in the mid-20th century, becoming the cornerstone in defense of egalitarian sexuality in the new science of sexology, and later on in evolutionary biology under the byproduct hypothesis. Recent research on the evolutionary origin of the female orgasm challenges this continuous trend of portraying female sexuality as only deriving from sexual homology. In linking the human female orgasm to females of other species, instead of taking male anatomy and pleasure as the sole reference for comparison, the female orgasm is individuated as a relatively autonomous evolutionary domain not necessarily coupled to reproductive functions. Moreover, current competing explanations of the evolution of female orgasm, which appear to contradict one another, may actually be reconcilable because they refer to different stages of the evolutionary history of the trait.

Our survey on the evolutionary explanations of female pleasure shows that the perception of the female orgasm as a mysterious, or even a paradoxical trait, is biased by cultural expectations about female pleasure. The expected link between sexuality and reproduction in women has been a core driving force of this riddle, and evolutionary biologists have further promoted this narrative by competing for a resolution to their created puzzle. Nonetheless, social biases in biological studies of the female orgasm do not only derive from the assumption of a tight link between sexuality and reproduction in women. Social expectations on the egalitarian nature of human sexuality have also played a role in characterizing as paradoxical the lack of reproductive function of the female orgasm. In breaking the necessary link between female pleasure and reproduction, new biological research on female orgasm also challenges the perception of biology as a source of essentialist associations between female sexuality and reproduction. These two dimensions of female physiology might be historically linked, but evolution itself broke this connection in our more recent history. As Halperin outlined decades ago, the paradigm of 'masculinity' was defined by the ability of men to isolate sexual pleasure and reproduction, even when only in men recreative and procreative sex are physiologically linked (Halperin, 1990, p. 285). Despite the fact that evolutionary hypotheses themselves do not imply any value statement about female sexuality (Lloyd, 2005; Wagner & Pavličev, 2016), unraveling the evolutionary origins of the decoupling between sexual pleasure and reproduction shows how biological theories of human sexuality do not only constrain but also provide new anthropological imaginaries for theorizing femininity.

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Silvia Basanta: Conceptualization; writing – original draft. **Laura Nuño de la Rosa**: Conceptualization; writing – original draft.

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PART II

EVOLUTION OF THE FETAL-MATERNAL INTERFACE

The placenta is the transient apposition of fetal membranes with the maternal tissue for physiological exchange (Mossman, 1991) and constitutes a contact organ between the mother and fetus. Placentation thus entails both placental and endometrial tissue. While the first mainly consists of trophoblasts from the fetal origin, the maternal compartment assembles a diverse array of pregnancy-specialized cells, including epithelial, endothelial, stromal, and immune cells, which participate in the development of the placenta and are actively involved in tissue remodeling, implantation, and immune tolerance.

Placentation is a crucial trait and one of the most variable structures in placental mammals. Therefore, it constitutes an ideal case study for researching the mechanisms underlying evolution and novelty. The fetal-maternal interface varies in several meaningful ways within placental eutherians, including the structure, branching morphology, and gross form of the main placenta and the presence of accessory placental structures (Mess & Carter, 2006). However, the detailed fetal-maternal interactions and the changes leading to the observed placental diversity still need to be better understood. Existing variation in invasiveness, which concerns the intimacy in cell-cell communication established between maternal and embryonic tissues, constitutes the most critical and exciting dimension of placental diversity. Quantified by the number of tissue layers separating maternal and fetal blood, the types of placentation in eutherian mammals have been traditionally classified into three groups: hemochorial, endotheliochorial, and epitheliochorial (Grosser, 1927).

In the most invasive form (hemochorial placentation), the fetal trophoblast cells directly contact maternal blood. During the development of a hemochorial placenta, placental cells replace the maternal endothelial cells lining uterine arteries and remodel them, a process known as endovascular invasion, and placental cells may also infiltrate into the endometrium up to the myometrial segments of the uterus, a process known as interstitial invasion. Phylogenetic inferences have inferred that invasive placentation arose in the stem lineage of placental mammals, with some debate still existing on whether the ancestral form was endotheliochorial (Mess & Carter, 2006) or hemochorial (Wildman et al., 2006; Elliot & Crespi, 2009). On the other hand, epitheliochorial placentation is the derived placental state (Vogel, 2005), having evolved independently multiple times within eutherians. Thus, some eutherian lineages present transitions towards less invasive, secondarily non-invasive placentation (Wildman et al., 2006).

The cellular changes accompanying changes in placental invasiveness are poorly understood. Changes could result from the recruitment or exclusion of specific cell types, the evolutionary origin or loss of cell types, or changes in gene expression. Trophoblast invasiveness strongly depends on its interaction with maternal tissue. The placental tissue of species with minimal invasiveness, such as the pig, expresses a highly invasive phenotype when transplanted into ectopic sites (Samuel & Perry, 1972; King, 1993), which indicates that the degree of placental invasiveness is not solely dependent on the trophoblast's ability to invade but also on the crucial role of the maternal endometrial reaction. The maternal uterine environment has undergone crucial evolutionary innovations concurrently with the evolution of implantation to facilitate trophoblast invasion, and at least one new maternal cell type evolved concurrently with invasive placentation: the decidual stromal cell (Wagner et al., 2014; Kin et al., 2016).

Species with less invasive placentation display a less developed decidual reaction (Ramsey et al., 1976). Decidualization, the differentiation process of endometrial stromal cells into decidual cells, happens when the blastocyst invades the endometrial stroma in species with hemochorial plantation. The absence of direct interaction between the trophoblast and maternal stroma in marsupial pregnancy makes decidualization a unique feature of eutherian pregnancy (Chavan et al., 2016; Wagner et al., 2014). The connection between decidualization and invasiveness is supported by the absence of

stromal decidualization in eutherian lineages that have evolved secondarily non-invasive placentation types (Carter & Enders, 2004; Carter & Mess, 2018). The deficiency or lack of decidualization in human conditions characterized by an abnormally increased placental invasion (in placenta *accreta*) or incomplete invasiveness (in preeclampsia) (Bartels et al., 2018; Conrad et al., 2017) further underscores the functional role of the stromal decidua in regulating trophoblast invasion by both promoting and restraining it.

Abundant research has been devoted to the origin and evolution of the uterine decidual stromal cell (DSC) (Brar et al., 2001; Kin et al., 2014; Lynch et al., 2008; Erkenbrack et al., 2018; Chavan et al., 2021). Beyond a new cell type, the decidua also constitutes an environmental niche that supports trophoblast invasion through vascularization and immune cell recruitment, transforming the endometrium into a vascularised, receptive tissue (Plaisier, 2011). The decidua is a complex tissue composed mainly of decidual and immune cells, including natural killer cells that assist in spiral artery remodeling (Croy et al., 2002; Wallace et al., 2012). Mammalian invasive placentation poses an immunological challenge to the maternal immune system (Moffett & Loke, 2006; Mor & Cardenas, 2010). Various mechanisms, such as the entrapment of the immune cells (Erlebacher, 2013), a cytokine shift towards Th2 type immunity (Wegmann et al., 1993), or the presence of pregnancy-specific immune cells, have been identified that contribute to fetal immunotolerance.

Overall, new cell types - fetal and maternal - and milestone developmental events, such as decidualization and endovascular and interstitial placental invasion, have underlined the evolution of the eutherian fetal-maternal interface. More granular knowledge of the precise cell types and signaling events underlying these developmental milestones have been enabled using transcriptomic, particularly single-cell transcriptomic methods, applied comparatively across species (Stadtmauer, Basanta, et al., 2024). Exploring these evolutionary changes in the mature placenta is the focus of **Chapter 3**, and the initial events of implantation are the focus of **Chapter 4**.

VARIATION IN THE FETAL-MATERNAL INTERFACE

❖ PHYLOGENY AND RECONSTRUCTIONS

From the very introduction of the term *Mammalian* by Carl Linnaeus in 1758 (Schiebinger, 1993) to an early consideration of the placenta as the representative organ to study evolution (Huxley, 1864), traits associated with female reproduction have been used to refer to and classify mammalian species (Hayssen & Orr, 2017). Thomas Huxley was the first to recognize the relevance of maternal decidualization to understanding evolutionary patterns (Pijnenborg & Vercruysse, 2004), later neglected in comparative studies that focused instead on the trophoblast.

The diversity of placental phenotypes across species has no parallel in other organs. The functional morphology of the mammalian placenta varies in meaningful ways, including variations in placental shape (discoid, diffuse, cotyledonary, zonary), internal structure (labyrinth or villi), interhemal barrier (from hemochorial to epitheliochorial), and the type of fetal membranes (the yolk sac placenta can be temporal, permanent, mission or inverted) present throughout eutherian gestation. Early evolutionary surveys of this diversity tended to conclude that invasive forms of placentation were derived. For instance, Turner (1876) suggested that the pig diffuse epitheliochorial placenta was the closest to the ancestral mammalian placenta. However, he ended up concluding that the placenta alone could not serve as the representative organ to explore phylogenetic relationships and that "characters drawn from more than one system of organs gives a safer guide to the affinities of an animal, than the existence, in only a single organ or system, of even a very close resemblance in form and structure" (Turner, 1876, p.53). In the same way, Grosser's classification of placental types based on the number

of tissue layers between fetal and maternal blood (Grosser, 1927) revived the debate over the implications of using the placenta to trace mammalian evolution. Grosser's suggestion that an increasing complexity towards more invasive forms of placentation happened in evolution was at odds with classifications of mammals based on other traits, leading to further disagreements on how to interpret placental variation (Luckett, 1993).

Later, these dimensions of variation became the cornerstone of comparative placentology and phylogenetic inferences, attempting to trace the evolutionary history of the mammalian placenta itself. The first surveys of molecular phylogeny focused primarily on the evolutionary history of the interhemal barrier (Carter, 2001), stating that an ancestral invasive placentation was followed by the secondary evolution of less invasive forms (Wildman, 2011). Likewise, evolutionary inferences suggested that the ancestral mammalian placenta had a labyrinthine structure, with villous placentas evolving multiple times independently from it (Wildman et al., 2006). However, the functional significance of most of the dimensions of placental diversity still needs to be discovered, not to mention its evolutionary patterns or the evolutionary forces behind it. The diversity concerning the interhaemal barrier, invasiveness, and its ancestral and derived patterns has attracted most of the attention.

❖ MODEL AND NON-MODEL SPECIES

The diversity of placentation and its underlying plasticity poses a challenge when it comes to finding animal models that can be informative for human placentation (Carter, 2007). Because of this, a substantial focus of paleontologists has been on finding animal models that better resemble, or resemble, in some aspects, human placentation (Carter & Mess, 2014; Mess, 2014). The placenta is still one of the least understood human organs, and animal models are crucial to countervail the restricted access to the human placenta as an organ of research (Guttmacher et al., 2014). Under this view, the human case is sometimes referred to as the most extreme case of invasive placentation (Pijnenborg et al., 1983), thus posing a different, more acute immunological challenge compared to any potential model species (Moffett & Loke, 2006). Comparative evidence, however, suggests that this form of human exceptionalism may be unwarranted.

Strepsirhine primates have noninvasive, epitheliochorial placentations (Luckett, 1974; King, 1984), whereas, within Haplorhini, New World primates present a lesser extent of trophoblast invasion in comparison to humans (Merker et al., 1987). Old World monkeys present the most similarities to the human condition (Carter & Mess, 2014), although whether trophoblast invasion is confined to the endovascular pathway (Blankenship et al., 1993; Enders et al., 2001) or extends through an interstitial route to the decidua is debatable (Enders & Blankenship, 2012). The crab-eating macaque (*Macaca fascicularis*) has been used in multiple placental and reproductive studies (Jiang et al., 2023). It shares with humans menstruation (Brenner & Slayden, 2012). Invasive endovascular trophoblast invasion but lacks interstitially invasive trophoblast, and pre-eclampsia has not been reported in this species (Moffett & Loke, 2006). Lastly, the gibbon and Great Apes present interstitial implantation and endovascular and interstitial routes of trophoblast invasion (Carter, 2007; Martin, 2007). However, the extravillous trophoblast invasion up to the inner third of the myometrium, as takes place in human placentation, is only known for the chimpanzee and the gorilla (Carter, 2011; Carter & Pijnenborg, 2011).

Beyond a possible resemblance to human placentation, animal models at different vital positions of the phylogeny are also essential to better understand the evolution of the fetal-maternal interface. Although studying rare and less conventional species is critical in tracing the evolutionary history of mammalian traits such as placentation and reproductive biology, comparative and evolutionary perspectives are scarce for many reasons. Most underrepresented species come with challenges and

limitations, from their difficult access (presence in the wild or status as protected species) to the many difficulties associated with non-traditional lab animal models. This problem is even more pressing when studying the fetal-maternal interface, as this type of research must deal with the complexities of mating and long months of gestation for many species. Still, animal models belonging to wildlife and non-traditional taxa could inform us about human placentation and the evolution of the fetal-maternal interface.

Afrotherian species constitute exciting case studies from a reproductive point of view. Afrotherian tenrecs belong to the family of Tenrecidae and are native to Madagascar. They possess unique reproductive features such as intrafollicular fertilization, cases of polyovulation (Nicoll & Racey, 1985), and reproductive features such as a cloaca (Riedelsheimer et al., 2007). Besides a labyrinth and junctional zone, their invasive placentas entail a haemophagaous region as an accessory placental structure (Carter et al., 2005). Tenrecs are generally considered to lack a decidua (Mess and Carter, 2006), although we have identified a putative pre-decidual cell type within the pregnant endometrium (Stadtmauer, Basanta, et al., 2024; **Chapter 3**).

Within Laurasiatheria, domestic ruminants and Carnivora are some of the best-known species regarding female reproductive biology and placentation (Carter & Mess, 2018). Although some are interesting because of their long gestations and precocial offspring (Berry & Anthony, 2008), Laurasiatheria species with epitheliochorial placentas are also important to better understand the repeated evolution of secondarily non-invasive placentations (Carter & Enders, 2013).

Within Euarchontoglires, rodents constitute the most commonly used animal models for female reproduction (Carter & Mess, 2014). Our knowledge of placentation largely derives from mice (Clark, 2014). The mouse placenta presents three anatomically distinct regions: the labyrinth, the junctional zone (with spongiotrophoblast and glycogen trophoblast), and giant trophoblast cells that penetrate the endometrium and maternal decidua. Trophoblast giant cells become polyploid through endoreduplication and constitute the most significant endocrine source of the placenta, underpinning the production of cytokines and hormones. Although sharing a hemochorial placentation type and fulfill analogous functions, human and murine placentas exhibit multiple structural and invasive differences (Georgiades et al., 2002). In the mouse, four different trophoblast lineages differentiate from progenitors at the junctional zone: invasive giant trophoblast cells, spongiotrophoblast, and glycogen cells. Mouse glycogen trophoblast cells are analogous to the human extravillous trophoblast, as they are involved in interstitial invasion and constitute an energy source (Ain et al., 2003). A subset of giant trophoblast cells remodel the maternal arteries, increasing maternal blood flow towards the placenta.

Muridae species possess a brief gestation with an altricial offspring that requires extensive parental care after birth and very short ovarian cycles. On the other hand, hystricomorph rodents have relatively long gestations and precocial newborns. These include the guinea pig *Cavia porcellus* and its relatives (Carter, 2007; Mess et al., 2007). The guinea pig labyrinth consists of a single layer of syncytiotrophoblast (Mess, 2003). Hystricomorph rodents have an accessory placental structure, the subplacenta, from which extraplacental cells analogous to the human extravillous trophoblast migrate and invade the decidua (Mess, 2003). The location of the subplacenta differs between hystricognathi species, and in guinea pigs, it is located at the roof of the central excavation of the placental disc. It comprises syncytiotrophoblast and cytotrophoblast layers and presents a layer of giant cells resulting from the transformation of the syncytial layer (Davies et al., 1961). Besides its function as a source of invasive trophoblast (Mess et al., 2007), the exact function of the subplacenta still needs to be better understood. It could be related to gonadotropin activity or the absorption of decidual proteins (Davies et al., 1961). In **Chapter 3**, I describe guinea pig placentation in more detail.

Outside placental mammals, marsupial models serve as outgroups for eutherian ancestral state reconstruction. They are thus essential to investigate the evolutionary transformations leading to the

evolution of invasive placentation and its ancestral mechanisms. The marsupial fetal-maternal interface usually comprises a yolk sac placenta that cannot break the maternal epithelium. Therefore, marsupials present an epitheliochorial type of placentation of a very different kind, called choriovitelline (except for some cases, such as the bandicoot, Padykula & Taylor, 1975) instead of chorioallantoic. Whereas eutherian embryos rely on hemotrophic nutrition from the transfer of nutrients from the maternal blood at the interhemal barrier, marsupial embryos rely more heavily on uterine gland secretions. Besides possessing a different type of epitheliochorial placentation, marsupials also lack the implantation process and instead undergo a short post-hatching attachment that soon leads to parturition. This implies that the duration of a true hemotrophic placentation phase is extremely short, especially when compared to the long eutherian gestation. Some authors have speculated on the possible evolutionary connections between marsupial post-hatching attachment and eutherian implantation, which I explore further in **Chapter 4**. The marsupial short attachment and eutherian implantation involve inflammatory reactions (Griffith et al., 2017; Mor et al., 2011), so it is plausible that eutherian implantation originated from controlling and modifying this inflammatory reaction into a subsequent stable, anti-inflammatory phase.

CELL TYPES, HOMOLOGY, AND CELL-CELL COMMUNICATION

Single-cell transcriptomics, which allows researchers to capture gene expression at the single-cell level, remains an active field and one of the most common tools used to identify and explore the heterogeneity of different cell types or cell populations in a given tissue, organ, or organism. Based on reducing the high-dimensional transcriptomic data into patterns of canonical gene signatures associated with cell clusters, single-cell transcriptomics has led to a new definition of cell types, mainly relying on the differential gene expression of marker genes, that has replaced morphological, histological, or functional characterizations (Zeng, 2022). Cell type definitions have been further reduced to the expression of a specific combination of transcription factors, known as a cell type-specific "combinatorial code" (Shirasaki & Pfaff, 2002) or molecular fingerprint (Arendt, 2005). Interestingly, although primarily data-based, this new definition sparked an awareness of cell types as the product of evolution (Vickaryous & Hall, 2006), and the new characterization is now inseparable from an evolutionary account of cell types, defined as a "set of cells in an organism that change in evolution together, partially independent of other cells, and are evolutionarily more closely related to each other than to other cells" (Arendt et al., 2016, pp.1-2). Under this light, cell types are individuated evolutionary units that can potentially undergo evolutionary changes without affecting other cell types.

Single-cell transcriptomics has led to a concept of cell types as cells clustering together in a gene expression manifold, similar to the definition of cell types as cells expressing the same orthologous genes for cell determination (Arendt, 2003). This identification of homologous cell types based on their underlying regulatory programs has led to a surge in comparative analyses, including comparing organism-wide cell atlases across different species (Wang et al., 2021), with numerous potential applications in investigating species-specific cellular innovations. Although the cross-species comparison of cell types is relatively new (Marioni & Arendt, 2017), it follows the underlying assumptions of examining the relations of the same morphological character across species and has emerged as a new variant of homology within evolutionary developmental biology (Arendt, 2005; Arendt et al., 2016).

Within this framework, some limitations and challenges remain. Homologous cell types might not show the highest mutual transcriptome similarity. For instance, as is the case for any phenotypic similarity, rooting cell type identity in the transcriptome cannot distinguish between similarity due to homology, convergence, or concerted evolution. Therefore, exploring the expression of shared

transcription factors known to play a role in cell type differentiation is crucial to ensure that similarity refers to evolutionary homology. Likewise, the identification of homologous cell types is also problematic due to the tendency of transcriptomics to co-evolve within organisms due to pleiotropy, a tendency known as species-signal (Liang et al., 2018), which challenges the assumption that cells belonging to the same type should have more similar gene expression than those of different kinds. Several tools have been designed to tackle identifying homologous cell types across species, including SAMap (Tarashansky et al., 2021), with application to the cell-type annotation of less well-known animal models.

Another layer of analysis deriving from single-cell technologies is inferring cell-cell communication. Under the assumption that the expression of ligands and receptors reflects the level of cell-cell communication, a pair of cell types is potentially communicating when one cell expresses the ligand and another the cognate receptor. Several methods and tools are available to explore cell-cell communication from single-cell data (reviewed in Armingol et al., 2021). These methods are employed to address the evolution of uterine signaling in **Chapters 3 and 4**.

❖ CELL TYPE HOMOLOGY AT THE FETAL-MATERNAL INTERFACE

Some challenges and limitations in identifying homologous and novel cell types across species are particularly challenging when studying placentation. First, trophoblast cell populations pass through developmental intermediate stages, making it hard to distinguish between different cell types of developmental stages within the species and between cell types sharing the same developmental versus evolutionary origin between species. Second, the trophoblast exemplifies how new definitions of cell types can clash with previous morphological or functional definitions due to the potential lack of correspondence between transcriptomic similarity and other phenotypic aspects. The intersection between the transcriptome and other phenotypic-based characterization of cell types is particularly relevant to the study of trophoblast evolution and diversity. Different trophoblast populations have been traditionally identified by their topological position or function (e.g., invasive) or other aspects of the phenotype, like fusion (syncytium) or ploidy (giant trophoblast cells). Linking these traditional sources of criteria to define trophoblast cell types to single-cell data is still a challenge.

Trophoblast cells can be divided into two groups depending on their anatomical position and specialized function shared across all eutherian species. On the one hand, trophoblast cells at the definite placenta specialize in the exchange between maternal and fetal blood. On the other hand, trophoblast cells close to the decidua basalis interact with maternal cells and facilitate an increased blood flow to the placenta. Another similarity across species is the division between cytotrophoblast and syncytiotrophoblast specialization. Whereas the first specializes in hormonal production and nutrient exchange, the cytotrophoblast gives rise to invasive cells.

Trophoblast cells belong to the same developmental lineage as placental epithelial cells (trophectoderm), differentiating from a common multipotent progenitor into distinct trophoblast sublineages with unique functions and morphologies. Cross-species study of cell types has traditionally differentiated transcription factors from other “effector genes” and prioritized transcription factors as better indicators of cell type identity (Wagner et al., 2014; Arendt et al., 2016). Trophoblast cells indeed have characteristic transcription factors within species, which have been well determined for mouse and human (Baines and Renaud, 2017). However, applying this approach to other species is complicated by a lack of conservation of many transcription factor regulators in the trophoblast. Trophoblast cells do not express exclusive transcription factors, as the ones identified are described in a wide range of other embryonic and adult cell types (Papuchova & Latos, 2022).

We understand cell types from gene expression, developmental lineage, and function. However, cell type homology still needs to be addressed in the case of the trophoblast and comparative studies

of cell type composition at the single-cell level. **Chapter 3** discusses the homology of trophoblast and decidual cells.

❖ SIGNALING AT THE FETAL-MATERNAL INTERFACE: A BIOLOGICAL INTERPRETATION OF CELL-CELL COMMUNICATION DATA

So far, there have been no comparative studies of inferred cell-cell communication from single-cell studies across species using the fetal-maternal interface as a case study. The origin of new cell types during the evolution of implantation and invasive placentation also entangles new cell-cell communication patterns. Although we have some knowledge of the evolution of cell types, it is unknown which new patterns and pregnancy-specific interactions evolved with time along the lineage of placental mammals. Reconstructing putative interactions between cells at the fetal-maternal interface and comparing them across species holds the promise of identifying some of the changes potentially involved in transitions towards more invasive or less invasive placentation regimes within eutherians.

Any comparative approach to inferred cell-cell communication from single-cell data, including the fetal-maternal interface, still requires us to know how to interpret this data meaningfully. Although many new tools and databases have been developed, the biological interpretation of the inferred ligand-receptor interactions between cell pairs remains challenging. A biological understanding of comparative cell-cell communication data requires engaging with theoretical and evolutionary questions dealing with more classic cases of homology and conservation.

Tissue types have traditionally been classified as assemblies of one given cell type, such as epithelial, connective, or nervous tissue. However, new models for interpreting single-cell inferred communication data would benefit from considering composite tissues assembled by different cell types. In particular, the signaling between the major cell type categories, namely epithelial, stromal, endothelial, and immune cells, has been claimed to constitute a basic organization unit (Adler et al., 2023). Vertebrate tissues would constitute variations around this general archetype, with the further diversification of new cell types and the evolution of new interactions among them. Cell categories and cell type pairs can also be thought of in connection with the types of signaling they are involved in. For instance, fibroblasts and macrophages would have a complementary signaling relation based on reciprocal interactions that maintain tissue homeostasis (Okabe & Medzhitov, 2016; Franklin, 2021). Mutual exclusivity, on the other hand, refers to those cell type pairs that cannot coexist in the same tissue compartment, as their signaling restricts the presence of another cell type. The disruption of this signaling relation could underlie pathological situations of cell overgrowth, such as metastasis or endometriosis (Adler et al., 2023). Thinking about invasive placentation from this frame could be productive. For instance, during the evolution of invasive placentation, the trophoblast needed to overcome signaling mutual exclusivity that prevented the presence of two different epithelial cell types: the maternal epithelium and trophoblast. Yet, it also had to establish reciprocal interactions with other maternal cells to ensure a stable placentation.

What could the intuition of a tissue archetype mean in an evolutionary context? To understand the concept of homology applied to higher levels of organization beyond the cell type, we need to refer to character identity mechanisms (ChIMs), namely those recognizable causal mechanisms, neither perfectly conserved nor completely variable, that underpin character identity (DiFrisco et al., 2020). The logic of character identity mechanisms applied to the functional units of interactions between cell types in a tissue applies the same principles that the homology paradigm for cell types. However, tracing tissue homology throughout phylogeny still requires much development at the theoretical and data analysis levels. For composite tissue types, the candidate for ChIMs will be those cell-cell interactions that maintain cell populations proportionately and guarantee tissue homeostasis (DiFrisco

et al., 2020). Even at the theoretical level, a distinction between general homeostatic signaling and signaling that can help track true homology can still be complicated.

We have mentioned cell categories based on their relationships with other cell types. The relational properties of cells can also be used to track homology or serve as marker genes. For instance, instead of focusing on transcription factors expressed in a cell-type-specific fashion, one could base analysis on the potential uniquely expressed ligands and receptors (Pavličev et al., 2017). The expression of ligands and receptors can also shed light on how stable the environment of a cell type is (Pavlicev & Wagner, 2024). For instance, the proportion of receptors for which the same cell type does not express the concomitant ligands could measure how autonomous or integrated the given cell type is in tissue. According to this, the more individualized cell types are, the more they will rely exclusively on autocrine signaling. The latter is predicted to happen in unstable environments.

In contrast, the less autonomous ones will depend on other cell types (allocrine or paracrine signaling), having more receptors or ligands that need cell-cell communication with a different cell type (Pavlicev & Wagner, 2024). This framework is also relevant for the case of the fetal-maternal interface, as it requires establishing allocrine communication between cell types that were previously not interacting, hence likely involving the replacement of some autocrine interactions by other cell types. Likewise, cells at the endometrium respond to hormonal changes and are subject to endocrine signaling and external factors that influence cell differentiation, thus having a more relational identity than cell types from less dynamic tissues. In addition, the degree of autocrine versus allocrine signaling can also be informed about its developmental stage, as cells in an early differentiation stage may be more involved in autocrine signaling.

Implantation initiates placental morphogenesis by first bringing both partner tissues into appropriate contact. The term is most widely used in species where the process involves invasion by the trophoblast, i.e., it is accompanied by degradation of the maternal host tissues. In invasive placentation, the syncytiotrophoblast must first displace, intrude, or fuse with the luminal epithelium to break into the stroma and breach the endothelium. The destruction of the endometrial epithelium is a loss of tissue integrity, typically triggering the wound response. Therefore, cell-cell interactions are disrupted and modified during the transition from endometrial homeostasis to establishing the decidua-placenta interface. The endometrium must have evolved to accommodate new cell types, maternal and fetal, into the tissue. The trophoblast as an epithelial cell and the decidua as a transformed stromal fibroblast both entail a new and different signaling potential but must also be integrated into the endometrial signaling network. Once the fetal-maternal interface is fully established, only some trophoblast subsets remain in close contact with maternal stromal, endothelial, and immune cells. These subsets are the ones undergoing either interstitial or endovascular forms of invasion, such as the invasive giant trophoblast cells (i-GTC), the human extravillous trophoblast (EVT), or the guinea pig extra subplacental cytotrophoblast (EPT). I explore this prediction in **Chapter 4**.

Cells at the fetal-maternal interface belong to different genetic individuals with different adaptive optima for variables such as nutrient allocation into the current versus future reproductive events. This fact is the basis of parent-offspring conflict theory (Trivers, 1974; Haig, 1993). While the fetal genes will be selected to enhance the transfer of nutrients, maternal genes will be selected to counteract this demand. This dynamic has been speculated to be a driving force for the evolution of viviparity and fetal-maternal interface diversity in vertebrates (Crespi & Semeniuk, 2004). During invasive placentation, the placenta releases hormones directly into the maternal circulation, and the dynamics of these interactions are thus predicted to be shaped by parent-offspring conflict (Haig, 1996). Some of the predictions of conflict theory are explored in **Chapter 3**.

Overall, the evolutionary analysis of cell-cell communication from high-throughput data is an emerging field in which little is settled. Part II of my dissertation presents work that explores how this can be applied to study mammalian pregnancy and provides a basis for future investigation.

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Cell type and cell signaling innovations underlying mammalian pregnancy

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Abstract

The decidual-placental interface is a key innovation of eutherian mammals. The origin of this chimeric fetal-maternal tissue poses a unique evolutionary puzzle. We present a multi-species atlas integrating single-cell transcriptomes from opossum (a marsupial), Malagasy common tenrec (an afrotherian), mouse and guinea pig (rodents) with previous macaque and human data (primates). We find that invasive trophoblasts share a transcriptomic signature across eutherians, likely representing a cell type family that radiated following the evolution of hemochorial placentation. Our data suggest that decidual stromal cells evolved stepwise from an immunomodulatory predecidual cell type found in *Tenrec*, followed by acquisition of an endocrine decidual cell in Boreoeutherian mammals. Finally, we recover evolutionary patterns in ligand-receptor signaling: Consistent with theoretical predictions, fetal and maternal cells show a strong tendency towards disambiguated signaling, however, a predicted escalatory arms race dynamic between them is restricted to few ligands. We reconstruct the uteroplacental cell-cell communication network of the eutherian common ancestor, inferring accelerated signaling loss in Euarchontoglires and accelerated gains in primates. Unexpectedly, fetal trophoblast cell types display strong integration into maternal signaling networks despite their recent evolutionary addition to the interface. Together, our results reveal a dynamic history of cell type origination and signaling co-evolution at the fetal-maternal interface.

Introduction

The origin of novel tissues and organs is essential for the evolution of complex multicellular organisms. However, understanding how evolution at higher levels of organization relates to evolution of cellular composition and signaling is currently limited. One of the most intense loci of cell signaling in the body is the fetal-maternal interface, where cells from the fetal placenta invade and establish communication with the maternal endometrium. Placental invasion, and the corresponding transformation of maternal tissues called decidualization, first evolved in the stem lineage of placental (eutherian) mammals[1,2]. In this paper, we trace the fetal-maternal interface to its evolutionary origin, and ask a fundamental question: how does a complex novelty, composed of highly interdependent constituent parts, come to be assembled in evolution?

Answering this question demands a comparative perspective. Single-cell transcriptomic atlases of the human fetal-maternal interface have yielded insight into the cell-cell interaction networks governing this chimeric organ[3–5]. However, the placenta is also one of the most rapidly evolving, and hence most diverse, characters among mammals. Here, we present and compare single-cell data from the mid-gestation fetal-maternal interface of five species bracketing key events in mammal phylogeny **(Figure 1a)**. These include the gray short-tailed opossum, *Monodelphis domestica*, a non-deciduate marsupial, and four eutherian species: the Malagasy common tenrec *Tenrec ecaudatus*, an afrotherian mammal considered to have a “primitive” form of hemochorial (invasive) placentation [10,11], and four Euarchontoglires - the guinea pig *Cavia porcellus*, the mouse *Mus musculus*, and recently published data from two primates *Macaca fascicularis*[7] and *Homo sapiens*[4,5].

We establish putative cell type homologies, identifying trophoblast populations of guinea pig and tenrec which share a gene expression signature with primate extravillous trophoblast, and discover a primitive “predecidual” cell type in the tenrec which suggests a stepwise evolution of the decidual stromal cell. We reconstruct ancestral cell-cell signaling networks, revealing the integration of novel cell types into the fetal-maternal interface. Finally, we test two long-standing theoretical predictions, the disambiguation hypothesis[8] and escalation hypothesis[9], at transcriptome-wide scale. In

so doing, we trace the co-evolutionary history of cell types and cell signaling across the origin and diversification of mammalian placentation.

Results

Therian mammals differ in placental and uterine cell type inventories

Single-cell transcriptomic data were collected from the uteroplacental interface of mammals at informative phylogenetic positions for the evolution of placentation **(Figure 1b)**. Samples were collected after placental development had completed but before the physiological changes leading to the onset of parturition. Datasets were analyzed following a standardized approach **(Extended Data 1)**. The resulting curated cell atlas **(Figure 1c)** consists of 404,118 cells and 145,153 nuclei totaling 549,271 libraries, with an average of 10,253 unique transcripts per library. Cell clusters were identified using Leiden clustering and annotated using marker gene expression and histology. To reduce over- or under-clustering, we used non-negative matrix factorization (NMF)[12] to identify coregulated gene modules **(Extended Data 2)**. Cell clusters lacking uniquely distinguishable NMF gene modules or markers were merged. Detailed cell type descriptions are provided as Supplementary Material.

Tissue organization **(Figure 1b)** and cell type composition **(Figure 1d)** vary across the mammals in our study. In eutherian mammals, both a vascular interface where maternal circulation is made available to the fetus and an interface where invading trophoblast contacts maternal decidua are present. The latter was the focus of analysis due to greater potential for co-evolved cell-cell signaling. In the opossum, however, fetal-maternal contact and nutrient exchange occur in the same location, a lamellar matrix of trophoblast which coats maternal epithelium **(Extended Data 3a)**. Uterine glands, which provide histotrophic nutrition, are expansive **(Figure 1b)**: more than 50% of the cells captured in the opossum were epithelial, of which 85% were glandular **(Figure 1d)**. Stromal cells such as fibroblasts are more scarce (8%) in opossum than in other species **(Figure 1b,d)**. In the tenrec, guinea pig, and mouse, the vascular interface is a trophoblast labyrinth surrounding maternal blood **(Figure 1b)**, whereas the human and macaque vascular interface consists of fetal villi reaching into a maternal blood sinus. In the tenrec, we identified glands containing mucus and leukocytes, suggesting continued histotrophic nutrition **(Extended Data 3b)**, whereas in the other eutherians histotrophy has ceased by the time placentation is complete. Lymphoid cells, primarily NK-like innate cells, constituted 58% of the total cells recovered in this species (**Figure 1d**), which contrasts with the opossum, where no uterine NK cells could be identified. The guinea pig, mouse, macaque and human have similar cell type compositions **(Figure 1d)**, but one notable structural difference: the guinea pig's interface is organized into a folded cytotrophoblast structure called the subplacenta, which anchors the placenta to the maternal decidua **(Figure 1b)** and gives rise to invasive trophoblast[13].

Assessment of cross-species homology between cell populations was assisted by self-assembling manifold mapping (SAMap)[14], which integrates cells from all species into a unified manifold **(Figure 1c)** while accounting for complex gene homology. Pairwise SAMap mapping scores between cell clusters **(Table S1)** formed reciprocally linked groups **(Extended Data 4)**. Conserved maternal cell types including smooth muscle, pericytes, leukocytes, endothelial, epithelial, and mesothelial cells showed consistent mapping across species. In contrast, trophoblast and decidual cell types displayed lower transcriptomic conservation across species, suggesting rapid cell type and gene expression evolution.

To obtain a global picture of cell type relationships preserving within-species hierarchical structure, we calculated a neighbor-joining tree of cell type transcriptomes. This yielded monophyletic

or near-monophyletic groupings corresponding to major cell type families **(Figure 1e; see Methods)**. The tree contains a hematopoietic cell clade with myeloid and lymphoid sub-groups, a clade of non-immune mesenchymal cells divided into one group consisting of fibroblasts, smooth muscle, and pericytes and the other of endothelial cells, and finally three mixed epithelial/trophoblast clades. These cell type family groupings were largely maintained when the genes used to generate the phylogeny were restricted to transcription factors **(Extended Data 5)**, suggesting that the pattern derives from regulatory identity and not solely phenotypic convergence. Rapid innovation of trophoblast and decidual cell types made one-to-one homology assessment complicated, so we explored their evolutionary history with more detailed analyses.

Placental cell types

Trophoblast cells are traditionally classified by topological location (e.g., extravillous), phenotype (e.g., glycogen trophoblast, syncytiotrophoblast), or ploidy (giant trophoblast), but whether these divisions correspond to cell type identities conserved across species is unknown.

Human trophoblast has been well documented by single-cell methods **(Figure 2a)**[5]: cytotrophoblast of the placental villi (Hs_VCT) can undergo fusion, becoming syncytiotrophoblast (Hs_SCT), or invade, becoming extravillous trophoblast (EVT). EVTs can migrate interstitially (Hs_iEVT) into the decidua, or endovascularly (Hs_eEVT) to replace maternal spiral artery endothelium. Macaque trophoblast divide into the same three major cell type groupings of cytotrophoblast of placental villi (Mf_iCTB), fused/syncytial trophoblast (Mf_fcCTB, Mf_SCT), and extravillous trophoblast (Mf_EVT)[7]. As not all mammals develop villi, we refer to other species' migratory trophoblast cells as "extraplacental trophoblast" (EPT).

In the opossum, two trophoblast populations were identified **(Figure 2a)**. Cytotrophoblast cells (Md_CTB) express transcription factors *WFDC2* and *ZBTB7C*, desmosome components *DSC2* and *DSG2,* as well as *HAND1*, detected via in situ hybridization in mononuclear cells of the trophectoderm **(Figure 2b)**. These cells also express the t-SNARE protein *SNAP25* involved in vesicle fusion[15], possibly related to fusion with maternal apocrine secretory bodies **(Extended Data 3a)**. The second population consists of syncytial trophoblast, which includes giant cells of the trophectoderm, termed syncytial knots[16] (Md_SCT) **(Figure 2b)**. Syncytial knots express *GCM1* and *DLX3,* cathepsins *CTSV* and *CTSH*, proteases *PRSS22* and *PRSS36*, high levels of the growth factor *VEGFA* and inflammatory mediators *IL1A, IL6, IL17A* and *PTGS2* as well as *PTGES*.

In the tenrec we identified four trophoblast populations **(Figure 2a)**. One cluster (Te_CTB-CYP11A1) expresses *CYP11A1*, the enzyme catalyzing the first step in steroid hormone production[17], which we localized to the superficial-most trophoblast coating the endometrium **(Figure 2b)**. These cells express heme oxidase *HMOX1*, consistent with reports in other tenrec species of catabolic breakdown of maternal hemoglobin by cytotrophoblast[18]. Steroid hormone-producing cytotrophoblast (Te_CTB-HSD3B1) expresses the progesterone synthase *HSD3B1* and the cortisol/cortisone converting enzyme *HSD11B1*. Non-steroidogenic cytotrophoblast cells are enriched for the somatotropin *IGF2* (Te_CTB-IGF2) as well as *WFDC2*. Finally, invasive extraplacental trophoblast (Te_EPT) expresses *ADAMTS18*, *PAPPA2*, *QSOX1*, and cell invasion regulators *SCAI*[19] and *ZEB1*[20]. Cells immunoreactive for the proteoglycan GPC1, a marker of invasive trophoblast[21] and angiogenesis[22], surround the deepest maternal vasculature **(Figure 2b)**, suggesting deep interstitial invasion. Unlike other eutherians, no expression of *prolactin* or other growth hormone paralogs was present in any *Tenrec* placental cell type.

In the guinea pig, seven trophoblast cell clusters were distinguished. Invasion is evident as $KRT7^{+}$ trophoblast cells are present in the decidua and surrounding maternal vasculature **(Extended Data 3c)**. Extraplacental trophoblast (Cp_iEPT) expresses *ADAM19*, *CNFN, FLT1, QSOX1,* and

GPC1 **(Figure 2a)**. GPC1^{+} cells surround maternal vasculature and reside in the myometrium **(Figure 2b)**. A population of trophoblast progenitors (Cp_CTB-CCC) share expression of proliferation regulators *DLX5*[23] and *HMGA1* with human cell column cytotrophoblast (Hs_VCT-CCC); indeed, cell aggregations in the guinea pig subplacenta have been identified as homologs to the human cell column[24]. Non-invasive cytotrophoblasts share the expression of the genes *CTSE, GJB2,* and *TMEM213* **(Figure 2a)**. Syncytiotrophoblast (Cp_SCT) expresses three paralogs of *prolactin*, transcription factors *TFAP2A and TFAP2C*, and *LCN2*, a gene linked to invasion in human EVT[25]. Syncytiotrophoblast "tongues", identified by elongate cell morphology combined with nuclear TFAP2C expression **(Figure 2b; Extended Data 3d)**, derive from subplacental cytotrophoblast and invade deeply into the decidua[13]. Coarse-meshed syncytiotrophoblast between the subplacenta and the labyrinth and giant cells between the subplacenta and labyrinth and invading the decidua, are present histologically **(Extended Data 3e)** but were not captured transcriptomically due to their size.

Mouse trophoblast cells were grouped into eight populations: spongiotrophoblast, syncytiotrophoblast, labyrinth cytotrophoblast, glycogen trophoblast, and three populations of giant cells **(Figure 2a)**. A subset of trophoblast are invasive, united by the expression of *Cdx2*, *Pcdh12*[26], *Plac1*, and *Tfap2c*. These include interstitially invasive glycogen trophoblast (Mm_GlyT), enriched in *Arid3a*, and their progenitors (Mm_GlyT_Prog), enriched for *Ascl2* and *Prdm1*[27]. Endovascularly invasive spiral artery-remodeling giant cells (Mm_TGC-SpA) express the prolactins *Prl2a1* and *Prl7b1*. Noninvasive cells of the labyrinth, canalicular (TGC-C) and sinusoidal (TGC-S) giant cells, express *Cited1*[28] and *Ctsj*[29] **(Figure 2a)**. Syncytiotrophoblast (Mm_SCT), in close proximity to maternal blood in the labyrinth, share some of these noninvasive cell markers and are also marked by *Slc16a1*, *Hsd11b2,* and the syncytin *Syna*[30].

Placental cell type homology inference identifies a eutherian radiation of invasive trophoblasts

We analyzed fine-grained relationships among trophoblasts by building a network of SAMap scores and identifying reciprocally linked cell clusters using Leiden community detection **(Figure 2c)**. The Leiden algorithm grouped the 35 total trophoblast cell populations into five communities which we considered putative homology groups. The resulting communities divided by degree of invasiveness, with one consisting of invasive cell types and three of noninvasive cell types **(Figure 2d)**.

The grouping of invasive (extraplacental) trophoblast was driven by genes demonstrated to regulate cell invasion, including proteases *ADAM19*, *MMP15*[31]*,* and *PAPPA2*[32], *ANXA4*[33]*, GPC1*[22]*,* and *PLAC8*[34] **(Figure 2d; Table S1)**. This group united extravillous trophoblast from human (Hs_EVT, Hs_eEVT/iEVT) and macaque (Mf_EVT) with rodent invasive cells (Mm_GlyT, Mm_TGC-SpA, Cp_iEPT) Opossum syncytial knot cells (Md_SCT) and extraplacental trophoblast from tenrec (Te_EPT) also fell into this group, suggesting deep conservation of this invasive placental gene signature across therians. Guinea pig SCT, the only syncytial cell type of the 6 species studied to have invasive properties, was also included in this homology group.

Three communities of noninvasive - primarily cytotrophoblast - cell types were also identified **(Figure 2d)**. Opossum cytotrophoblast (Md_CTB) exhibited linkage to cytotrophoblast-like cells of all other species **(Figure 2d)**: tenrec cytotrophoblast (Te_CTB-HSD3B1), guinea pig subplacental progenitors (Cp_CTB-CCC), and human villous cell column progenitors (Hs_VCT-CCC). The similarity of the earlier developmental stages of human and guinea pig cytotrophoblast to marsupial cytotrophoblast suggests that eutherian-specific placental cell types may have evolved from a conserved cytotrophoblast cell type.

In contrast to invasiveness, other phenotypic traits of cells, such as fusion and endoreduplication, did not consistently sort into SAMap communities **(Figure 2d)**. Human giant cells (Hs_TGC), which develop from deeply migratory extravillous cells[5], mapped to extraplacental cells of other species rather than to giant cells of the mouse. Furthermore, placental cell types which undergo cell fusion, "syncytiotrophoblast", did not show cross-species conservation except between human and macaque, the two species in our study with the most recent evolutionary divergence **(Figure 2c)**. These formed a homology community with mouse spongiotrophoblast, driven by expression of large numbers of pregnancy-specific glycoproteins, although independent evolution of *PSG* and *CEACAM* gene families in rodents and primates[35] suggests that cell type homology based on expression of these genes is unlikely. While giant opossum knot cells (Md_SCT) are histologically syncytial, their gene expression differs from other therian trophoblasts by inflammatory and growth factor production. Likewise, guinea pig SCT clustered with other invasive cell types rather than with noninvasive mouse or human SCT. We conclude that, with the exception of macaque and human SCT, syncytial trophoblasts and giant trophoblast cell types most likely arose independently in major mammalian lineages.

Overall, the patterns we observe are consistent with eutherian invasive trophoblasts having descended from a single cell type with invasive potential in the therian common ancestor. This cell type was evidently retained in *Tenrec* and *Monodelphis,* which both have only one cell population belonging to invasive homology groups. This ancestral extraplacentally migratory cell likely radiated into interstitially migratory and artery-remodeling subtypes in Boreoeutherian mammals, and acquired distinct morphological phenotypes in different lineages such as endoreduplication. Conservation of a suite of invasion-enhancing genes, including matrix proteases and metastasis drivers **(Figure 2d)**, suggest that an ancient potential for cellular invasion was elaborated upon during this process.

Cross-species decidual cell diversity and a predecidual cell type in *Tenrec*

Decidualization is a coordinated transformation of the endometrium during pregnancy, including arterial remodeling, recruitment of tissue-specific leukocytes (NK cells), and differentiation of endometrial stromal fibroblasts into epithelioid decidual stromal cells. Decidualization is histologically observed only in mammals with hemochorial placentation[1]. Molecular mechanisms of decidual development are poorly known outside of humans and mice, but regulatory interactions essential for decidual cell development, such as protein-protein interactions between HOXA11 and FOXO1, are shared among eutherian mammals and lacking in marsupials[36,37]. Using our transcriptomic atlas, we investigated whether the decidual stromal cell is indeed a unitary cell type novel to eutherians and whether the gene-regulatory signature of decidual stromal cells is conserved across eutherian mammals.

Recent single-cell studies[4,5] suggest greater human decidual stromal cell diversity than the single prolactin-producing cell type canonically recognized in the field[38]. We used matrix factorization to identify co-regulated gene sets within the human endometrial stroma: this identified gene expression modules corresponding to the three decidual cell populations annotated by Vento-Tormo and colleagues[4] **(Extended Data 2; Table S2)**. The gene expression module of "Type I" stromal cells (Hs_dS1) includes contractility- and myofibroblast-related genes *ACTA2* and *TAGLN* and myosin light chain kinase *MYLK*. The "Type II" (Hs_dS2) gene expression module includes *IL15*, *HAND2, MEIS1, FOXO1*, and *LEFTY2.* These genes are upregulated during the initial wave of human decidualization[39] and commonly called "predecidual"[40]. The "Type III" (Hs_dS3) gene expression module includes *IGFBP1* and *PRL*[38]*,* as well as *WNT4* and the decidual proteoglycans *DCN* and *LUM*; we refer to these as "endocrine decidual cells". Comprehensive sequencing of stromal cells across the human menstrual cycle found that predecidual cells generated during the secretory phase in the absence of an

embryo, or spontaneous decidual cells, are *IL15*$^{+}$ *PRL*$^{-}$ [41], similar to dS2 of pregnancy, not dS3. Thus, human *IL15*$^{+}$ *PRL*$^{+}$ endocrine decidual cells are pregnancy-specific, whereas *IL15*$^{+}$ *PRL*$^{-}$ predecidual cells also develop spontaneously during the proliferative phase of the menstrual cycle. The decidua of the pregnant macaque, an Old-World Monkey with similar reproductive characteristics including menstruation, shows the same three stromal cell types: *ACTA2*$^{+}$ Mf_dS1, *IL15*$^{+}$ *PRL*$^{-}$ Mf_dS2, and *PRL*$^{+}$ *IGFBP1*$^{+}$ Mf_dS3.

In the opossum, two stromal cell populations are present. Both express *FOXO1*, *HOXA11,* estrogen receptor *ESR1* and progesterone receptor *PGR* **(Figure 3a)**. Endometrial fibroblasts (Md_eS1) are enriched for *SMOC2*, *WNT5A,* and the relaxin receptor *RXFP1*, and reside in the stroma surrounding endometrial glands[36]. The other population (Md_TF) are enriched for *FBLN1*, *CLEC3B*, *MFAP4*, and are present in the myometrium **(Figure 3b)** and we thus consider them tissue fibroblasts. 29% of Md_eS1 cells express *HAND2* and *IL15*, markers of human predecidual cells thought to be eutherian-specific[42], but these cells did not group together consistently with sub-clustering. This suggests that *HAND2 and IL15* expression is a developmentally accessible gene-regulatory state even in stromal cells of the opossum, but became stabilized into a persistent cell type identity only during eutherian evolution.

In the tenrec, two major endometrial stromal cell populations are present, united by expression of *HOXA11* and *HOXA9* **(Figure 3a)**. HOXA11 is localized to peri-glandular endometrial stroma **(Figure 3b)**, like the opossum's Md_eS1 cells. RNA velocity[43] analysis inferred a developmental trajectory from fibroblast-like (Te_dS1) to predecidual-like (Te_dS2) cells **(Figure 3c-d)**. Tenrec dS1 are enriched in *CLEC3B***,** *COL14A1, C7*, and *SMOC2,* whereas predecidual cells express hormone receptors *ERBB4*, *RXFP1, ESR1* and *PGR*, decidual transcription factor *KLF7*, and predecidual markers *HAND2* and *IL15*. IL15 is a chemokine for uterine NK cells[4], with the potential to act via binding a receptor (*IL2RB+IL2RG*) expressed in tenrec uterine NK cells. Treatment of uterine stromal cells isolated from the pregnant uterus of *T. ecaudatus* with deciduogenic stimuli cAMP and MPA for 6 days elicited upregulation of these predecidual markers **(Figure 3e)**, consistent with the predecidual cells observed *in vivo* representing this species' full decidual response. The discovery of a cell population in *Tenrec* resembling *IL15*$^{+}$ *HAND2*$^{+}$ human predecidual cells and lack of a clear endocrine decidual cell suggests that in this member of the basally divergent mammalian clade Afrotheria, uterine stroma persists in a state similar to the human predecidua of menstruation, rather than developing endocrine decidual cells.

The guinea pig decidual stroma contains one ECM-remodeling and two endocrinologically active populations. All three express *HOXA11*, *HAND2, ESR1, PGR,* and the rodent-specific decidual marker *HOPX*[45] **(Figure 3a)**. Endocrine decidual cells include a cluster enriched in oxytocin *OXT* (Cp_dS3-OXT) and another expressing a *prolactin* gene (Cp_dS3-PRL) surrounding maternal spiral arteries **(Figure 3b)**. The majority (84%) of stromal cells, however, are marked by expression of *SMOC1*, *SMOC2*, and *WNT5A*. Based on their size **(Figure 3b)**, expression of the cell cycle gene *E2F8* implicated in endoreduplication[46], and previous descriptions of giant decidual cells in necrotic areas of guinea pig decidua[13], we identify these as giant decidual cells, and based on SAMap affinity and lack of an endocrine profile, classify them as a Type II cell type (Cp_dS2).

The mouse day 15.5 decidua consists of a single endocrine cell type (Mm_dS3) marked by the prolactin paralog *Prl8a2*, *Pgr*, *Esr1*, and *Hand2*. This cell type also expresses 6 genes recently discovered to have maternally-biased parent-of-origin imprinting[47] – *Adamts5*, *Erv3*, *Mfap5*, *Tfpi2*, *Tnfrsf11b*, and *Tnfrsf23* **(Figure 3a)**: these genes are tumor suppressors[48,49] and may represent mechanisms to regulate trophoblast invasion. A subpopulation of decidual cells show reduced expression of *Pgr, Esr1, Hand2,* and *Hoxa11* but maintain expression of stress-related genes, including the chaperone *Cryab*, the inflammatory enzyme *Ctsk*, and the oxidative stress gene *Gpx3*. These we term progesterone-resistant decidual cells (Mm_dS3-Pgr^{-}). They have a matching gene

expression profile with a *Pgr*$^{-}$ oxidative stress-enriched endocrine decidual cell population which a previous study identified as postmature decidua[50], and share similarities with so-called senescent *PGR*$^{-}$ endocrine decidual cells of the human which exist in balance with normal endocrine decidual cells[51]. Cells corresponding to the predecidual cells of the tenrec and human were absent in the mouse.

Stepwise evolution of the uterine decidua in Eutheria

While our cell type phylogeny **(Figure 1e)** identified high-level family groupings, it could not resolve one-to-one decidual cell type homologies. We investigated fine-scale decidual cell relationships using SAMap community detection and comparison of gene co-regulatory modules across species.

Two stromal cell type communities in the SAMap network were highly distinct from other mesenchymal cell types, smooth muscle cells and placental fibroblasts **(Figure 3f)**. One community consists predominantly of endocrine decidual cells and the other of contractile fibroblasts and predecidual stromal cells. The endocrine or "Type III" community united the two endocrine decidual cell populations of the guinea pig (*OXT*$^{+}$ and *PRL*$^{+}$), mouse *Pgr*$^{+}$ and *Pgr*$^{-}$ decidual cells, and human and macaque *PRL*$^{+}$ *IGFBP1*$^{+}$ cells. The predecidual and non-decidual stromal cell community consisted of the opossum's *SMOC2*$^{+}$ eS1 and *FBLN1*$^{+}$ tissue fibroblasts, tenrec dS1 and predecidual cells, guinea pig dS2, mouse tissue fibroblasts, endometrial stromal fibroblasts from macaque and human, and macaque predecidual cells. Human predecidual Hs_dS2 had strong mapping affinities to both the predecidual cells of other mammals and to endocrine decidua and were formally assigned to the same group as endocrine decidual cells. This result was robust across all iterations of community detection attempted, although we suspect that this more reflects the fuzziness of drawing hard boundaries in cells undergoing a continuous developmental process than it does an evolutionary difference between the predecidua of humans and other mammals.

We next looked for conservation of co-regulated gene modules in decidual cells of our 6-species comparison **(Extended Data 2)**. A hierarchically-clustered correlation matrix of gene expression programs identified by NMF analysis grouped them into two families **(Figure 3g; Table S2)**: Predecidual-like gene-regulatory modules from all species tended to weigh highly *MEIS1, WT1, ESR1*, *PGR,* and *EGFR*, and were active in human, macaque, tenrec, and guinea pig. Endocrine gene-regulatory modules, which had high weightings of *PRL, LUM*, *DCN*, *VIM*, *PPIB*, and *DUSP1*, were active in endocrine decidua of all eutherians (mouse, guinea pig, human and macaque), but no counterpart was identified in the opossum. Predecidual and endocrine gene expression modules showed low or negative correlation with one another **(Figure 3g)**, meaning that genes expressed in the predecidual cells tended not to be expressed in mature decidual cells **(Extended Data 2)**. We interpret this as evidence that predecidual cells and endocrine decidual cells are robustly distinct alternative gene-regulatory states.

Integrating these lines of evidence leads to the following model for decidual cell evolution **(Figure 3h)**. Un-decidualized, contractile endometrial fibroblasts, or "Type I" cells, have homologs across therian mammals. These cells predominate in the non-pregnant uterus. Eutherians display decidual stromal cells falling into two states – predecidual or "Type II" decidual cells, which secrete immunomodulatory peptides, and "Type III" decidual cells, which secrete endocrine and growth-regulatory signals. Type II decidual cells likely represent a novel eutherian stromal cell type, and are evolutionarily modified from mesenchymal cells of the ancestral therian endometrium by upregulation of *HAND2* and *IL15*[52]. In the two rodents sampled, no stromal cell type produces *IL15*; instead, decidual *IL15* is solely produced by macrophages. However, the deep phylogenetic age of the tenrec-human divergence suggests that the *IL15*$^{+}$ predecidual cell type was lost in rodents, or is restricted to early stages of pregnancy. These findings suggest that decidual stromal cell diversity

expanded within crown Placentalia in two steps – establishment of the decidual stromal cell type in its initial predecidual *IL15*$^{+}$ *PRL*$^{-}$ state, followed by later evolution of the endocrine (*PRL*$^{+}$) decidual cells observed in rodents and primates.

Co-evolutionary dynamics of cell communication

We next asked to what degree the cell-cell communication between fetal and maternal cell types shows evidence of co-evolution. We inferred signaling between cell types from complementary secreted ligand-receptor gene expression and traced the evolutionary history of these interactions within Theria.

Invasive trophoblast cells are integrated into the endometrial signaling network

The evolution of placentation is predicted to result in increased functional integration of fetal and maternal cells[53]. As an approximation of functional integration, we compared the number of non-autocrine ("allocrine") signaling interactions each cell type engages in **(see Methods)**. Cells producing more allocrine ligands tend to also receive more allocrine signals themselves (Pearson's $p < 0.05$ in all species except *Monodelphis*), and degrees of allocrine ligand production varied across major cell type families (one-way ANOVA $p < 0.05$ in all species except *Cavia*; **Figure 4a**). Stromal cells, particularly decidual stromal cells, display the greatest degrees of allocrine signaling. Invasive trophoblast (EVT/EPT, Mm_GlyT in mouse and Md_SCT in opossum) also show high degrees of allocrine signaling **(Figure 4a)**. Macrophages and endothelial cells rank intermediately, whereas eutherian syncytiotrophoblast and immune effector cells (BC, TC, NKC, PMN) show the fewest allocrine interactions **(Figure 4a)**. *Monodelphis GCM1*$^{+}$ trophoblast and the human Type III decidual cell have the highest Kleinberg[54] hub scores in their respective signaling networks, whereas lymphoid cells from all species rank among the lowest **(Extended Data 6a)**. We interpret these findings to mean that fetal and maternal cell types that make direct contact have evolved to function as signaling hubs, highly integrated into the signaling network of the fetal-maternal interface, whereas peripheral immune cells without a stable niche do not evolve integration to the tissue microenvironment[55].

Reconstruction of common ancestral fetal-maternal signaling

We inferred the fetal-maternal signaling of common ancestors in our tree using maximum-parsimony ancestral state reconstruction on ligand and receptor expression of pooled fetal and maternal cell types **(Table S4)**. We identify a total of 83 signals which the placenta of the first viviparous mammal likely sent to its mother, including relaxin *RLN2*, platelet-derived growth factors *PDGFA* and *PDGFC*, prostaglandin E2 via *PTGES3*, and *TGFB1* and *TGFB2* **(Figure 4b)**. We traced gains and losses of signaling interactions throughout mammal evolution by comparing ancestral states. At the Placentalia node, an additional 44 signals were inferred to have been gained, including vascular growth factors *VEGFB* and *VEGFC* and the insulin-like growth factor *IGF2*. To gain more precise insight, we reconstructed full ancestral signaling networks between major cell type classes **(Table S4)**. The reconstructed network of the Placental mammal common ancestor revealed that early eutherian trophoblast had already acquired a signaling potential towards maternal stromal, vascular, epithelial, and immune cells **(Figure 4c)**.

Human, macaque, mouse, and guinea pig belong to the clade Euarchontoglires. Their stem lineage evolved 4 fetal-maternal signals absent in the basally branching *Tenrec,* including *CCL2* and *CCL4*, and also lost fetal expression of 11 ligands, including cytokines *IL1A* and *TNF* and neutrophil chemoattractant *CXCL2*, suggesting that inflammatory signaling by the placenta was suppressed after the divergence of tenrec and opossum. The two rodents in our sample, *Cavia porcellus* and *Mus musculus,* share an additional 10 ligands, including *FGF7*, *IL34*, *OSM* (related to *LIF*), and *prolactin*.

Many fetal-maternal ligand-receptor interactions are species-specific. *Mus musculus* saw an expansion of novel *CEACAM* genes, and the midgestation guinea pig placenta is unique in *OXT* production, produced only at parturition in other species. The human lineage saw the acquisition of novel *growth hormone* paralogs, and the *Monodelphis domestica* placenta is inferred to have gained expression of additional pro-inflammatory cytokines.

We calculated rates of evolutionary change in fetal-to-maternal signaling using a time-calibrated mammalian tree[56] **(Figure 4b)**. The greatest rates of evolutionary change were in the stem lineage leading to Euarchontoglires, with as many as 1.25 changes/Myr, many of these being losses of inflammatory signaling (e.g. *IL1A, PTGIS, CXCL2, TNF*) by fetal cell types. High rates of change were also observed in both primate lineages. In contrast, the lineage leading to *Monodelphis domestica* and the lineage leading to *Tenrec ecaudatus* show slower turnover. These findings suggest that the diversification of Euarchontoglires entailed an accelerated evolution of fetal-maternal communication compared to basally branching placentals and marsupial lineages.

Fetal and maternal signaling show disambiguation

A model of placental hormone evolution based on evolutionary signaling theory [8] predicts that fetal-maternal signaling systems will evolve towards maternal-only or fetal-only expression to reduce fetal manipulation of maternal physiology. Selection against fetal and maternal co-expression of ligands would manifest as a pattern of "disambiguation". To quantify disambiguation, we compared the observed number of fetal-only and maternal-only expressed ligands from different signaling families (e.g. WNT, NOTCH) against a null statistical model based on random distribution of ligands to cell types regardless of origin **(Figure 5a-b; see Methods)**. In all species, fetal and maternal co-expression of ligands was lower than expected by our null model **(Figure 5b)**. The most consistently disambiguated ligand families across species included WNTs, steroids, FGFs, and various families of immunomodulatory factors **(Figure 5b; Table S3)**.

Gene duplication can increase disambiguation by the creation of novel placenta- or decidua-expressed paralogs. In the murine lineage, the *prolactin* gene family includes 26 members and a strongly disambiguated expression pattern with 10 fetal-specific paralogs **(Figure 5c; Table S3)**. In human and macaque, duplication of the *GH1* locus has led to the origin of various placenta-specific paralogs, whereas the somatoropin *PRL* is maternal-only **(Figure 5c)**. Cell type-specific growth factors involved in core tissue homeostasis, such as *VEGF*, *PDGF,* and *CSF1*, showed overwhelmingly bilateral expression **(Table S3)**.

Extreme production of the angiogenic factor *VEGFA* by the opossum trophoblast can be interpreted as a nutrient-soliciting signal. The disambiguation model[8] predicts that in an evolutionary stable state, the mother should silence her own copy or utilize a different VEGF paralog. However, all 3 VEGF paralogs in the opossum were co-expressed. This may be explained by developmental constraints countervailing the selection for disambiguation, due to the importance of VEGF in tissue homeostasis. Alternatively, disambiguation may be achieved by maternal utilization of a splice variant of VEGFA with differential signaling properties, as documented with the $VEGF^{111}$ isoform in the marsupial *Sminthopsis crassicaudata*, the rat, and viviparous skinks[57].

Escalation of fetal-maternal signaling

Parent-offspring conflict over nutrient allocation during pregnancy is predicted to lead to an asymmetric evolutionary arms race[58] in which gains to fetal signaling strength are matched by reduced maternal responsiveness[9]. We tested for correlated fetal ligand-maternal receptor co-evolution at the transcriptomic level by comparing reconstructed evolutionary changes in ligand and receptor

expression across internal branches of the tree. We tested for a quantitative escalation pattern without complete suppression by modeling changes in expression levels as continuous traits and testing for phylogenetically-independent[59] associations between ligands and cognate receptor expression **(Figure 5d; Table S5)**. Inferred evolutionary changes along internal branches of the tree did not show a global prevalence towards escalation regardless of whether all secreted signaling peptides are considered or just growth factors (gene ontology group GO:0008083), **(Extended Data 6b-c; Table S5)**. Across the tree, negative ligand-receptor correlations were observed for the inhibin-activin pair INHA–ACVR1B+ACVR2A (p = 1.1 x 10^{-3}) and the well-studied gene pair IGF2–IGF1R[60] (p = 2.7 x 10^{-2}) **(Figure 5d)**. Other pairs such as VEGFA–FLT1 showed positive correlation (p = 5.6 x 10^{-3}), i.e. gains to both fetal ligand and maternal receptor expression along the same branches, possibly reflecting a cooperative trend towards greater placental vascular development in multiple eutherian lineages rather than a manipulation-desensitization dynamic.

In the branch leading from the human-macaque ancestor to humans, for which the ancestral state confidence was high due to the sampling of two rodents and two outgroups, only the growth factors *PGF* and *TGFB3* showed asymmetric escalation **(Figure 5e)**, whereas *PRL*, *KITLG*, and *IGF2* were inferred to be undergoing de-escalation relative to the human-macaque ancestor. When modeling ligand and receptor expression as binary on/off traits using expression thresholding, lineages in which more fetal ligands were gained saw more gains in maternal receptors, rather than losses **(Figure 4b)**. These findings suggest that only a select subset of placental-maternal signaling is subject to antagonistic gene expression evolution. That said, our analysis cannot exclude antagonistic dynamics at the post-transcriptional level, as in the case of decoy receptors[60] or the chemical properties of signal peptides such as their half-life[61].

Discussion

The eutherian fetal-maternal interface is the product of three major evolutionary events – the origin of endometrial decidualization, the evolution of a distinct placental organ (in contrast to simple apposition of fetal and maternal tissue), and the origin of trophoblast invasion[2]. The latter is extreme in species with deep interstitial invasion such as humans and guinea pigs. In this paper, we have compared the cell type composition and cell-cell signaling networks of species spanning these transitions revealing a complex history of cell type and cell signaling evolution.

Cross-species mapping suggests that eutherian mammals underwent an expansion in trophoblast cell type diversity. We infer that the therian ancestor, like the extant opossum, had two trophoblast cell types, one homologous to eutherian cytotrophoblast and the other specialized in signaling to the mother with weak invasive potential. Extraplacental trophoblast of tenrec, guinea pig, macaque and human and spiral artery-remodeling trophoblast in the mouse shared a common gene signature. The grouping of phylogenetically distant trophoblast transcriptomes by invasive potential suggests that the invasive/noninvasive phenotypic divide may reflect an ancient divide in trophoblast cell type identity, rather than convergent evolution.

Endometrial stromal cells fall into homology groups of different phylogenetic ages, suggesting a stepwise accrual of decidual cell diversity in eutherians. The discovery of a primitive *IL15*$^{+}$ predecidual cell in *Tenrec*, with gene expression similarity to human Type II decidual cells as well as endometrial stromal cells of other species, suggests that the predecidual cell is conserved over the 99 million year *Tenrec-Homo* divergence. Endocrine (*PRL*$^{+}$) decidual cells likely arose later in eutherian evolution, restricted to humans and rodents in our sampling (Euarchontoglires) and lacking in *Tenrec*; investigation of decidua in more mammals from the basally-branching Afrotheria and Xenarthra clades will allow this evolution to be more thoroughly untangled.

Our findings are consistent with a model of cell type evolution playing out at two distinct levels: broad transcriptomic "cell type identities" and functional gene expression modules or "apomeres"[62], a division established in neuronal[63] and photoreceptor[64] evolution. In addition to novel cell types of decidual stroma and extraplacental trophoblast, fetal and maternal cells both show evidence of acquisition of functional apomeres. On the fetal side, syncytiotrophoblasts are transcriptomically dissimilar across species, and perform diverse functions - inflammatory and growth factor signaling in the opossum, invasion in the guinea pig, vascular interface in the mouse, and gonadotropin production in the human. Molecular genetic evidence suggests that trophoblast cell fusion is associated with five independent insertions of retroviral envelope genes in the opossum, tenrec, guinea pig, muroid, and primate lineages[65–67], a pattern congruent with our inferred lack of homology between syncytiotrophoblast beyond the human-macaque pair. Likewise, prolactin expression in endocrine decidual cells is associated with independent transposable element insertions upstream of the *prolactin* promoters in rodents and primates[68]. These findings suggest that both trophoblast and decidual evolution have involved parallel evolution of new phenotypes, like cell fusion and hormone production, by recruitment of retroviral and transposable elements into gene regulatory networks of these cell types. Identifying which of the cell populations we identify here correspond to developmentally robust cell type identities, and the factors determining their differentiation, will require further targeted investigation.

Compelling theoretical models have long been advanced for the co-evolutionary signaling dynamics between the fetus and mother[9]. Our test of the disambiguation hypothesis[8] revealed consistent and strong overrepresentation of both fetal-specific and maternal-specific ligand repertoires. This pattern was driven in part by recent gene duplication into fetal-specific paralogs[35]. A logical next step would be to investigate genetic mechanisms, such as capture of ligand genes by fetal-specific enhancers[69]. The escalation hypothesis predicts that increased fetal ligand production should be complemented by reduction in corresponding receptor expression by the mother. Only a select few ligand-receptor pairs in our sample consistently followed this dynamic, suggesting it does not hold for placental-uterine signaling universally; however, some of the outlier ligand-recetor pairs we identified are plausibly involved in escalation such as *IGF2*. Finally, we used ancestral state reconstruction to trace the co-evolutionary history of ligand-receptor signaling, reconstructed the cell signaling interactions of the Placental common ancestor, and found that Boreoeutherians underwent a more rapid divergence in fetal-maternal signaling than the opossum and tenrec lineages. This implies that fetal-maternal co-evolution was not solely concentrated at the origin of eutherian placentation; rather, the pace of gene-regulatory innovation in mammals has even accelerated in eutherian evolution, including substantial changes in recent primate and rodent evolution.

We acknowledge limitations to inferring ligand-receptor signaling from RNA expression. The potential for false negatives due to sequencing drop-out, incomplete genome annotation in non-model species, and protein-protein interaction divergence likely inflate the inferred number of species-specific signaling innovations. Differences in gestation length and placental physiology between our six species may limit the comparability of the chosen gestational stages for trophoblast and decidua, which are developmentally dynamic. Nevertheless, the outlines of a multistage history of cell type and cell phenotypic evolution can clearly be seen.

The fetal-maternal interface has attracted attention in recent years as a model system for single-cell genomics and ligand-mediated cell signaling at scale[3-5]. The atlas we present here demonstrates that the field has reached the point where long-standing evolutionary questions about parent-offspring co-evolution can be addressed at a resolution heretofore impossible. The molecular genetic mechanisms behind signaling innovation, integration, and disambiguation will require future investigation. As high-throughput genomics of non-traditional model species becomes more

technologically accessible, expanded taxon sampling will allow the kinds of phylogenetic reconstructions we introduce here to expand in precision and scope.

The evolutionary approach we introduce for cell-cell communication holds potential for broader application. Comparative analysis may help uncover the co-evolution of cell types within other tissues, and in other inter-organismal symbioses, an avenue opened by recent advances in single-cell transcriptomics of multicellular parasites[70] and commensals[71].

Data Availability

Sequencing data have been uploaded to the NCBI Gene Expression Omnibus at accession GSE274701. Human gene expression data used in this analysis were retrieved from https://www.reproductivecellatlas.org/ and macaque data from the NCBI accession GSE180637.

Code Availability

The analytical code for this study is available at https://gitlab.com/dnjst/fmi2024. Additional processing scripts for cell-cell communication data are archived in a python package at https://gitlab.com/dnjst/chinpy.

End Notes

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Author Contributions

D.J.S, S.B., G.P.W., and M.P. designed the study. G.P.W., M.P., F.vB., D.J.S. and S.B. secured funding. D.J.S., S.B., J.D.M., A.G.C. and G.P.W. conducted experiments. G.R.S., G.D., J.D.M., D.J.S. and S.B. maintained animal colonies. D.J.S. and S.B. conducted analysis. D.J.S. and S.B. drafted the manuscript. G.P.W. and M.P. edited the manuscript. All authors provided comments and approved the manuscript before submission.

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Materials and Methods

Animal Husbandry

Pregnancy samples were taken at gestation points after the establishment of the definite placenta and, in the case of guinea pigs, before the luteal-placental shift in progesterone production. Time points chosen were in opossum day 13.5 of 14.5 total gestation, in tenrec day 28-29 of 56, in guinea pig day 30.5 of 62, and in mouse day 15.5 of 19.5 days gestation. These were integrated with the human atlas generated from 4-13 weeks, roughly approximate days 28-91 out of a 280-day gestation, and macaque samples generated from stages ranging from 20-62 of a 162-day gestation.

M. domestica were raised in a breeding colony at Yale University according to established technical protocols(*1*) and ethical protocols approved by the Yale University Institutional Animal Care and Use Committee (#2020-11313). Male and female animals were housed separately after 3 months of age, at which point female opossums were introduced to the male room for sexual preconditioning, and breeding was attempted after 6 months. During breeding, non-cycling female opossums were introduced to the male room for 1 day, and then subsequently swapped into the used cage of a prospective male partner for 5 days. After this period, both individuals were placed into a breeding cage and video recorded to assess the time of copulation. If multiple copulations were observed, the first was always used to calibrate 0 dpc; samples were taken one day before parturition at 13.5 dpc (n=2).

T. ecaudatus were maintained in a breeding colony at the University of Nevada, Las Vegas according to approved University of Nevada, Las Vegas Institutional Animal Care protocols. Individuals in the colony descend from a population of 40 wild-caught animals imported from Mauritius in June 2014. Animals were mated and mid-gestation was sampled for scRNA-seq between 28 and 29 days (n=3) following the first exposure of females to males.

C. porcellus (Charles River) were maintained at the University of Vienna according to Institutional Animal Care protocols, on standard chow and water ad libitum. The estrus cycle was monitored by examination of vaginal membrane opening and the animals were mated in estrus at 3-4 months of age, and video recorded to detect copulation. Midgestation samples (n=2) were collected 30.5 days post copulation.

M. musculus (C57BL/6J) were maintained at the University of Vienna on standard chow and water ad libitum according to University Institutional Animal Care protocols. Animals of age 2.5-4 months were used for the experiments. Estrus cycle was monitored by vaginal swabbing, and the females were mated when approaching estrus. Copulation was determined by the presence of a copulatory plug. The start of gestation is counted from the midnight of the night preceding the detection of copulatory plug. Midgestation samples (n=2) were collected 15.5 days post copulation.

Single-Cell RNA Sequencing

Uteri were dissected into phosphate-buffered saline (PBS), separated from the cervix and fallopian tube, and opened longitudinally. Embryos and directly attached extraembryonic membranes were removed via severing of the umbilical cords. In the guinea pig, the labyrinth was removed prior to sample preparation. Tissue samples were kept at 4°C for approximately 30 minutes until they could be dissociated into a single-cell suspension. Due to necessity, *Tenrec* samples were shipped in RPMI medium on ice overnight and dissociated the next day.

All tissue was processed as follows, with small species-specific modifications as noted. Tissue was minced with a scalpel into ~1 mm^3 cubes in 2 mL of digestive solution containing 0.2 mg/mL

Liberase TL (05401020001, Sigma). Tissue suspensions were heated at 37°C for 15 minutes and then passed 10 times through a 16-gauge needle attached to a 3-mL syringe. This incubation and needle passage process was repeated two more times, with the substitution of an 18-gauge needle. Finally, 2 mL of charcoal-stripped fetal bovine serum (100-199, Gemini) was added and the suspension was immediately passed through a 70-µm cell strainer then a 40-µm cell strainer. The flowthrough was pelleted by centrifugation at 500 g for 5 minutes and cells were resuspended in 1x ACK red blood cell lysis buffer (A1049201, Thermo-Fisher), incubated at room temperature for 5 minutes, centrifuged again and resuspended in PBS containing 0.04% bovine serum albumen (A9647, Sigma). At this point, cells were examined on a hemocytometer to gauge concentration and check for debris and cell death using trypan blue stain (15250061, ThermoFisher).

Mouse tissue was processed as above with the addition of Accumax (Stem Cell Technologies) to the final 0.04% bovine serum albumen solution. Guinea pig tissue was processed as with the mouse with the following modifications: a wide-bore 1 mL pipette tip was substituted for the 16-gauge needle, and 0.2 mg/mL collagenase I (17018029, Thermo-Fisher) was added to the digestion solution. As tenrec and guinea pig tissue showed higher cell death, a fractionated protocol was adopted to reduce exposure of cells to digestive enzymes. For these species, cells which had dislodged following each passage step were separated from intact tissue and immediately passed through a 70-µm cell strainer into 2 mL of charcoal-stripped fetal bovine serum (100-199, Gemini), while remaining intact tissue was allowed to continue with further digestion. Each fraction was centrifuged at 300 rpm for 5 minutes and resuspended in 500 µL ACK red blood cell lysis buffer (A1049201, Thermo-Fisher) for 5 minutes, then centrifuged at 500 rpm for 2 minutes and resuspended in 0.04% bovine serum albumen (A9647, Sigma) and kept on ice until dissociation was complete. Cells were counted on a hemocytometer and recombined in a proportional manner before sequencing.

Cells were captured using the 10X Chromium platform (3' chemistry, version 3), libraries were generated according to manufacturer protocols (CG000315). Libraries were sequenced using an Illumina NovaSeq by the Yale Center for Genomic Analysis (*M. domestica and T. ecaudatus*) and at the Next Generation Sequencing Facility of Vienna Biocenter (*C. porcellus and M. musculus*).

Laser Microdissection

Microbulk transcriptomes were generated from laser-microdissected cryosections of 13.5 dpc bilaminar omphalopleure of the opossum (n=3) to verify that pro-inflammatory putative fetal cells were indeed placental in origin. Samples for this procedure were dissected into PBS and subsequently immersed Tissue-Tek OCT compound (Sakura Finetek, 4583) in a block mold and flash frozen by exposure to a bath of isopentane surrounded by dry ice, and stored at -80°C. 14-µm sections were prepared from the resulting blocks on a cryomicrotome (Microm HM 500 OM) and placed onto polyethylene napthalate membrane slides (Leica Microsystems, 11505158) which had been exposed to ultraviolet irradiation in a biosafety cabinet (Baker SterilGARD, SG-404) for 30 minutes. Sections were processed on a Leica LMD7000 apparatus and excised tissue dropped directly into a lysis buffer for processing using a Qiagen RNeasy Micro Kit (74004). RNA libraries were prepared by the Yale Center for Genomic Analysis using a NEBNext Low Input Library Prep Kit (E6420) and sequenced using an Illumina NovaSeq. Reads were aligned to the transcriptomes used for scRNA-seq analysis using kallisto (0.45.0)(*2*).

Histology and Histochemistry

Histological samples were fixed for 24 hours in 10% neutral buffered formalin, followed by dehydration to 70% Ethanol and kept at 4°C (days-weeks) or -20°C (months) before paraffin embedding. Staining with Gill 2 hematoxylin and Y alcoholic eosin was performed according to(*3*).

Bright-field images for Figure 1 were taken with a Leica Thunder Imager (*Tenrec*), a Nikon Eclipse E600 (*Monodelphis*), and an EVOS M7000 (*Cavia* and *Mus*).

Immunohistochemistry was performed using antibodies recorded in **Table S6**.

For chromogenic immunohistochemistry, slides were incubated for 1 hour at 65°C, dewaxed with xylene, washed in 100% ethanol, and rehydrated in running tap water. Antigen retrieval was performed for 1 hour in a 95°C vegetable steamer containing 10 uM sodium citrate (pH 6.0). Slides were washed in phosphate-buffered saline and blocked with 0.1% mass/volume bovine serum albumen. Peroxidases were blocked by incubation in 0.03% hydrogen peroxide containing sodium azide (DAKO) in a humidification chamber for 30 minutes. Slides were incubated overnight at 4°C in primary antibody solutions, blocked, treated with horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. Finally, slides were washed in blocking solution once more and treated with 3,3'-diaminobenzidine (DAKO K401011-2) for 5 minutes and counterstained with Gill 2 hematoxylin before brightfield imaging.

For fluorescent immunohistochemistry, the procedure had the following modifications. Peroxidase blocking and 3,3'-diaminobenzidine was not used in favor of secondary antibodies, conjugated with the fluorophore. Images were acquired using the EVOS M7000 Imaging System (Invitrogen). Fluorescent images were also captured in the red channel and superimposed to leverage the autofluorescent nature of erythrocytes to highlight the location of vasculature.

In Situ Hybridization

Single-molecule fluorescence *in situ* hybridization was conducted on formalin-fixed paraffin-embedded sections using the RNAscope platform following the manufacturer's protocols (ACD Bio 323100-USM for fluorescent and 322310-USM for chromogenic). Probes were designed against the equivalent transcript sequences used for RNA-seq alignment and deposited into the RNAscope database as standardized probes. Probes used are listed in **Table S7.**

Fluorescent RNAscope was performed using the MultiPlex platform (ACD Bio 323100) with Opal 520, Opal 570, Opal 620, and Opal 690 fluorophores (Akoya Biosciences). Fluorescent images were captured using a laser scanning confocal microscope (Leica Stellaris 8 Falcon). For Figure 3b, Fluorescent images were also captured at 690 nm and superimposed to leverage the autofluorescent nature of the opossum glands to highlight the location of glandular epithelia. Chromogenic RNAscope was performed using the RNAscope 2.5 HD Brown Assay (ACD Bio 322300) and imaged on an EVOS M7000 microscope.

Single-Cell Data Analysis

Reads were obtained in FASTQ format from the respective sequencing cores. *M. fascicularis* reads were downloaded from the NCBI Gene Expression Omnibus (GSE180637), and *H. sapiens* aligned counts were downloaded from https://www.reproductivecellatlas.org.

Reads were aligned to reference genomes using the 10X Genomics CellRanger software (≥v7.0.0). *Monodelphis domestica*, *Cavia porcellus*, *Mus musculus*, and *Macaca fascicularis* were mapped to their respective Ensembl genome annotations (ASM229v1 v104, cavPor3 v104, GRCm39 v104, and Macaca_fascicularis_6.0 v112, respectively). For *Tenrec ecaudatus* a novel unpublished genome was provided by the laboratory of Michael Hiller and Fauna Bio, annotated using the TOGA pipeline(*4*).

Quality control included filtering cells with low count (less than 700 unique features for maternal cells and less than 500 unique features for fetal cells) or high mitochondrial gene expression (more than 25%), followed by doublet detection by scrublet (v0.2.3)(*5*) and doubletdetection (v4.2)(*6*), to remove clusters consisting of majority doublets. Library size normalization, log1p

normalization, feature selection, and dimensionality reduction were performed using scanpy ≥ v1.9.1(*7*), with the exception of the mouse, which was annotated using equivalent functions in Seurat v4(*8*) and exported as raw counts and then reprocessed in scanpy. Principal components were adjusted to correct for batch effect across biological replicates using harmony (harmonypy, v0.0.9, r-harmony, v0.1)(*9*). UMAP embeddings were calculated and Leiden(*10*) clustering was performed to partition cells into putative cell types. Marker genes were calculated based upon differential expression (logistic regression, Wilcoxon rank-sum test, and t-test as implemented by scanpy.pp.rank_genes_groups) and used to annotate groups. Cells were annotated using a combination of these markers and active genes belonging to NMF gene expression modules (see below), and cell clusters lacking uniquely distinguishable NMF gene modules or markers were merged. Fetal cells were identified by a combination of placenta-specific marker gene expression, and non-overlap with non-pregnant uterine samples. In the case of the tenrec which was sufficiently outbred, single nucleotide polymorphism-based inference of the genome of origin using souporcell (v2.5)(*11*) **(Extended Data 1)**. Clusters of cells which souporcell assigned to the genome of lesser abundance (putative fetal) overlapped with those annotated as fetal by differential gene expression. Spliced and unspliced transcript calling was performed using the velocyto package (v0.17.17)(*12*) and RNA velocity was modeled using scVelo (v0.30)(*13*).

Because the ground-truth database was curated from the human literature and insufficient data exist to curate ligand-receptor pairs for each species, the non-human transcriptomes were converted into “human-equivalent” transcriptomes by mapping of genes to their top human ortholog as detected by BLAST of translated peptide sequence(*14*). To maximize coverage, in cases of many:one human orthology, counts of all detected paralogs were pooled together. This transformed expression matrix was used only for cell communication analysis and cell phylogeny inference. Dimensionality reduction, cluster identification, differential expression testing, and marker gene identification and plotting were conducted on unadulterated species-relevant gene sets.

Cell Type Phylogeny

A phylogenetic tree of cell type transcriptomes was calculated using cell type-aggregated transcriptomes from all species, with orthology resolved using the human BLAST method described above. Clusters with high annotation uncertainty, extremely low capture rate, or possible artifactual nature ('Md_LE-OAT', 'Te_T-gd', 'Md_PMN-NE', 'Mm_TC-NKT', 'Mm_NEU', 'Te_NEU-CHL1', 'Hs_Granulocytes', 'Mm_fMES-Myoblast', 'Mm_fFB-ribo'), with high erythroid signature ('Mf_Mega_progenitor', 'Mf_Mye_progenitor', 'Mf_e&ysEry2', 'Mf_e&ysEry3', 'Mf_EMP', 'Mf_BP'), and those originating from the embryo proper ('Mf_LP._Meso', 'Mf_Al', 'Mf_PS', 'Mf_PGC', 'Mf_Node', 'Mf_EPI') were excluded from analysis. Gene expression matrices were corrected for “species signal”(*15*) by linear regression to remove the species of origin covariate from cell transcriptomes with the scanpy.pp.regress_out function. A euclidean distance matrix between cell type transcriptomes was then calculated and a neighbor-joining tree generated using the TreeMethods python package (v1.0.3; https://github.com/BradBalderson/TreeMethods). An alternative tree was also generated using the same approach with the additional subsetting of genes to only those annotated as transcription factors in Lambert et al.(*16*). Cartoons of cell types were modified from BioRender, Erkenbrack et al.(*17*), DiFrisco et al.(*18*), and original art.

Homology Inference by SAMap

SAMap (v.1.3.4)(*14*) was used to calculate gene homology-aware similarity scores between cells of different species. BLAST (v2.14.1)(*19*) graphs were generated from the ENSEMBL

proteomes of respective species, with the exception of *Tenrec ecaudatus* for which the transcriptome was used with tblastx mode. SAMap mapping tables were exported and analyzed using hierarchical clustering (seaborn, v0.13.1) as well as network analysis (networkx, v3.2.1)(*20*). For network analysis, SAMap mapping scores were used as edge weights and cell types as nodes. Communities were detected using the Leiden algorithm (leidenalg, v0.10.2)(*10*). Stability of the resulting communities was assessed by repeating community detection with a range of 10000 random initial states and constructing a co-occurrence matrix between cell types: cluster stability was defined as the mean of all co-occurrence values between members of the final community.

Gene Expression Program Identification and Cross-Species Comparison

cNMF v1.4.1(*21*) was used to infer gene expression programs from count matrices of each species. Analysis was performed following a modified protocol from(*22*). Optimal numbers of factors (K) were chosen based upon manual examination of stability-error curves over a range of possible K values from 10-50, selecting a value immediately preceding a sudden drop in stability score **(Extended Data 2)**.

For cross-species comparison, spectra scores from cNMF (i.e., gene loadings in NMF factors) were used. Non-human genes were mapped to their closest human orthologs as determined by BLAST score (see above), and in cases of many-to-one mapping, the highest loading score was used. Pearson correlation was calculated between programs and hierarchical clustering conducted using the clustermap function in seaborn (v0.13.1).

In Vitro Decidualization of Primary Tenrec Stromal Cells

Mesenchymal stromal cells were isolated from the midgestation *Tenrec ecaudatus* uterus by differential attachment. Cells were expanded in T25 flasks in a growth medium consisting of 15.5 g/L Dulbecco's modified eagle medium (30-2002, ATCC), 1.2 g/L sodium bicarbonate, 10 mL/L sodium pyruvate (11360, Thermo Fisher), and 1 mL/L ITS supplement (354350, VWR) in 10% charcoal-stripped fetal bovine serum (100-199, Gemini), and then seeded into 12-well plates (3.9 cm^2) for 1 day. Samples were treated with either base medium (Base), 1 μM medroxyprogesterone acetate (MPA; M1629, Sigma-Aldrich), or 1 μM MPA and 0.5 mM 8-bromoadenosine 3',5'-cyclic adenosine monophosphate (cAMP; B7880, Sigma-Aldrich) for 6 days, with replenishment on day 3 (n=4 for each condition, total n=12). Bulk RNA was isolated using a Qiagen RNeasy Micro Kit (74004), libraries were prepared by the Yale Center for Genomic Analysis and sequenced using an Illumina NovaSeq. Reads were aligned to the draft genome using kallisto (v0.45.0). Significance of differentially-expressed genes was calculated using the Wald test function of pyDESeq2 (v0.4.10)(*23*) with multiple test adjustment using the Benjamini-Hochberg method.

Cell-Cell Communication Analysis

Putative cell interactions were inferred using expression thresholding, followed by statistical testing for significantly cell type-enriched interactions. A ground truth ligand-receptor database was built as a manually extended fork of CellPhoneDB v5.0.0(*24*) with additional curation and metadata. This modified list is archived at https://gitlab.com/dnjst/ViennaCPDB/. From these transcriptomes and the ground-truth list, interaction scores for all cell type pairs were generated using the LIANA+ (v1.2.0)(*25*) and chinpy (https://gitlab.com/dnjst/chinpy; v0.0.55) frameworks.

For co-evolutionary integration analysis, all ligand-receptor interactions in the ground truth database were classified into "off" (ligand and receptor off), "allocrine ligand" (ligand on, receptor off), "allocrine receptor" (receptor on, ligand off), or "autocrine" (ligand and receptor on) states,

based upon an expression threshold of 20% of cells in a cluster. Classification of ligands was done in this manner to measure intrinsic signaling potential for each individual cell type in a way which is not inflated by the number of clusters of other cell types identified in the tissue, in contrast to network analysis, where the splitting of a given cell type into multiple clusters inflates the number of inferred outgoing interactions for other cell types. Statistical associations between cell class and number of allocrine ligands were assessed using a one-way analysis of variance. Kleinberg hub and authority scores(*26*) were calculated for all cell types within their respective species signaling networks using the HITS algorithm as implemented in the hub_score() and authority_score() functions in igraph (v0.11.6), with edge weights set to the LIANA+ "expression products" of each inferred interaction (product of ligand and receptor log-normalized transcripts per 10,000).

For disambiguation analysis, a manually-corrected version of CellPhoneDB "classification" metadata was used to group ligands into ligand families. To preserve gene duplication events, in cases of many-to-one homology, all possible homologs to the human ground truth ligand list were included in the target species' ligand family using Ensembl (mouse, guinea pig, opossum) or BLAST (tenrec) orthology. Expected values of disambiguated ligands were calculated as described in Statistical Analysis.

For discrete ancestral state reconstruction, interactions were scored in a boolean on/off manner based on a nonzero expression threshold of 10% of cells in a cluster for all subunits of both ligand and receptor. Maximum parsimony was calculated using the castor package (v1.8.0)(*27*). Edge changes were calculated for all internal branches of the 5-species tree by identifying genes considered "on" in descendant nodes but inferred "off" in the immediate common ancestor node, or vice versa.

For continuous ancestral state reconstruction, restricted maximum likelihood via the ape package (v5.8)(*28*) was used to estimate ancestral states. Expression values for continuous analysis were in natural log-transformed transcripts per 10,000 scale (scanpy.pp.log1p). To reduce artifacts, only genes expressed in at least 4 of 5 species were subjected to continuous ancestral state reconstruction (i.e., one loss/absence of a gene was tolerated). Changes in continuous expression values for ligands and receptors along internal branches of the 5-species tree were calculated by subtracting descendant node expression values from inferred immediate ancestral node values.

For escalation analysis, phylogenetic independent contrasts(*29*) (PIC) were calculated for ligand and corresponding receptor expression values (scaled as above) using the pic() function in ape (v5.8)(*28*). Linear regression was performed on the PIC values using the model ligand PIC ~ receptor PIC + 0. That is, with ligand PIC as the independent variable and receptor PIC as the dependent variable, with the intercept forced to zero (assuming that when there is no change in ligand expression, there is no change expected in receptor expression). P-values reported as "PIC P-value" result from a two-sided t-test on the estimated slope coefficient of linear regression of ligand versus receptor PICs. Linear regression was also performed on observed expression values to return a regression slope.

Statistical Analysis

For cell-cell communication inference, single-cell transcriptomes from multiple biological replicates (sample sizes reported above) were pooled before inference of cell communication was performed, following common practice(*30*). Instead of biological replicate-based testing, other statistical tests, such as CellPhoneDB(*31*) and CellChat(*32*) permutation tests, are standard; output from these tests (calculated as described above using LIANA+) are available for ligand-receptor interactions of all species as **Supplementary Data**. Likewise, the sample size of scRNA-seq studies complicates statistical testing of differential cell type abundance(*33*), so the relative abundance measures in **Figure 1d** are presented as pooled values.

Disambiguation of ligands was compared to a null model in which the probability of randomly redistribution of ligands to cell types leading to exclusively maternal or exclusively fetal expression is assessed. First, all secreted peptide and small molecule-mediated ligand-receptor interactions were classified into “fetal-only”, “maternal-only”, or “both” expression groups based on the threshold value of 20% or more of the cells sequenced expressing the ligand in. Ligands were grouped into ligand families using an additionally curated version of the CellPhoneDB (v5.0.0) “classification” metadata. For each ligand family, the per-cell type probability of a given ligand being expressed was calculated according to the equation:

$$P_{on} = \frac{L_{expressed}}{S_{pathway} \times (N_{fetal} + N_{maternal})}$$

where $L_{expressed}$ is the observed number of “on” calls within the ligand family across fetal and maternal cell types, N_{fetal} is the number of fetal cell types, $N_{maternal}$ the number of maternal cell types, and $S_{pathway}$ the number of ligands in the family.

The null probabilities of fetal-only and maternal-only expression for a ligand, respectively, were given by:

$$P_{fetalonly} = \left(1 - (1 - P_{on})^{N_{fetal}}\right) \cdot (1 - P_{on})^{N_{maternal}}$$

$$P_{maternalonly} = \left(1 - (1 - P_{on})^{N_{maternal}}\right) \cdot (1 - P_{on})^{N_{fetal}}$$

The expected fetal-only and maternal-only ligands in each family were obtained by multiplying $P_{fetalonly}$ and $P_{maternalonly}$ by the number of ligands in the family.

Finally, a one-way chi-squared goodness of fit test for identity of observed and expected counts of the 3 scoring categories (coexpressed, fetal-only, and maternal-only) was used to obtain chi-squared and p-values, with individual observations corresponding to ligands within a family. The Benjamini-Hochberg correction was used (statsmodels.multitest.multipletests v0.14.1) with an alpha of 0.05 to obtain false discovery rate q-values. For plotting, ligand families were considered significant if $p \leq 0.05$ and $q \leq 0.05$. Bonferroni-corrected p-values, obtained by multiplying *p* by the number of ligands in a family, are also reported in **Table S3**.

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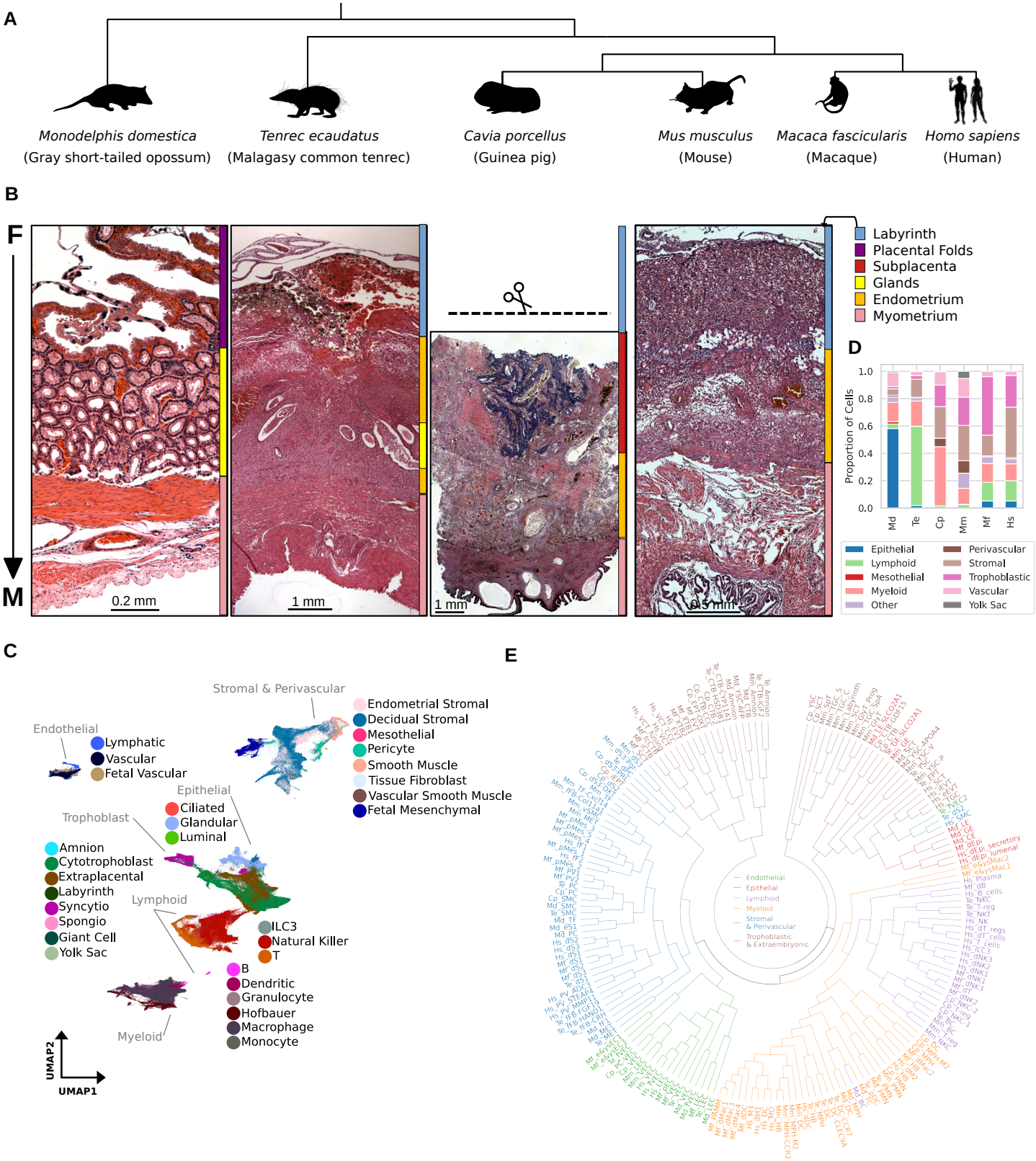

Figure. 1. Single-cell transcriptomic atlases of five mammalian species spanning the diversification of viviparity – from left to right: *Monodelphis domestica*, *Tenrec ecaudatus, Cavia porcellus, Mus musculus, Homo sapiens*. **(A)** Species used in the study. **(B)** Histology of the fetal-maternal interface of the species in (a). Color bars reflect tissue regions (legend on right) from fetal (F) to maternal (M). **(C)** Unified cross-species UMAP (SAMap), colored by cell type, with prominent divisions annotated in gray. **(D)** Proportional abundances of cell types belonging to major cell type families in each sample. **(E)** A neighbor-joining phylogenetic tree showing hierarchical relationships of cell clusters recovered, colored by broad families. For a full list of cell type descriptions, see Supplementary Material. CTB: cytotrophoblast; DC: dendritic cell; dS1/dS2/dS3: decidual stromal cell; eS1: endometrial stromal fibroblast; EPT: extraplacental trophoblast; iEPT: invasive extraplacental trophoblast; EVT: extravillous trophoblast; eEVT: endovascular extravillous trophoblast; iEVT: interstitial extravillous trophoblast; fFB/fMES: fetal mesenchymal cell; GE: glandular epithelial cell; HB: Hofbauer cell; ILC: innate lymphoid cell; LE: luminal epithelial cell; LEC: lymphatic endothelial cell; MET: mesothelial cell; MO: monocyte; MPH: macrophage; NK/NKC: natural killer cell; PC/PV: pericyte; PMN: polymorphonuclear cell; SCT: syncytiotrophoblast; SMC: smooth muscle cell; SpT: spongiotrophoblast; TC: T cell; T-reg: regulatory T cell; TF: tissue fibroblast; TGC: trophoblast giant cell; TGC-SpA: spiral artery-remodeling trophoblast giant cell; GlyT: glycogen trophoblast cell; GlyT_Prog: glycogen trophoblast progenitor; TGC_S: sinusoidal trophoblast giant cell; TGC_C: canal trophoblast giant cell; VEC: vascular endothelial cell; fVEC: fetal vascular endothelial cell; YSC: yolk sac cell; YSC-P: parietal yolk sac cell; YSC-V: visceral yolk sac cell.

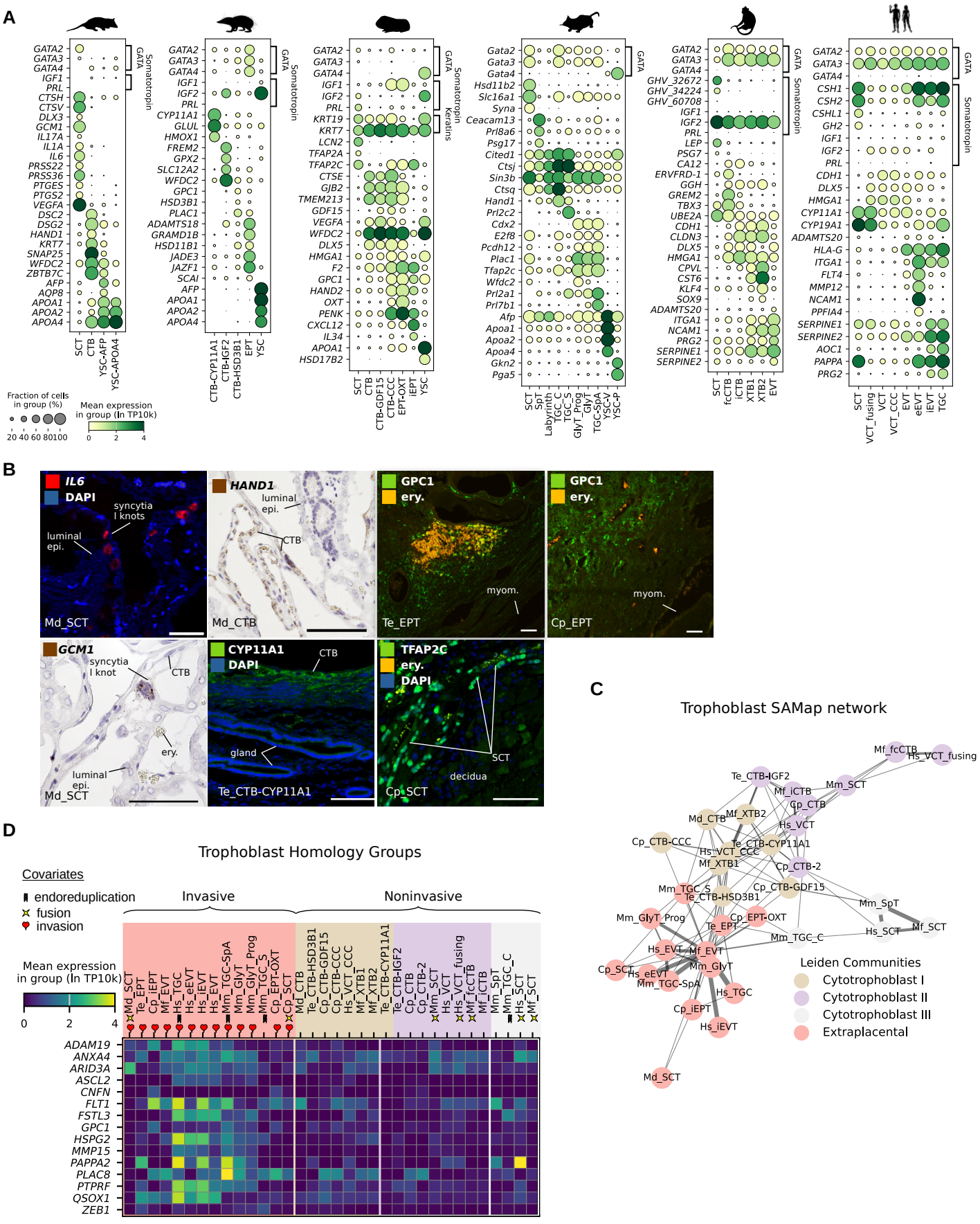

Figure 2. Trophoblast cell types and homology inference. **(A)** Expression of trophoblast marker genes. SCT: syncytiotrophoblast; CTB: cytotrophoblast; EPT/EVT: extraplacental/extravillous trophoblast; YSC: yolk sac cell; TGC: giant cell; CCC: cell column. **(B)** Histological localization of select cell type markers from (a). epi.: epithelium; myom.: myometrium; ery.: erythrocytes. Scale bars = 100 µm. **(C)** Network of SAMap linkages between trophoblast cells, with edge thicknesses are proportional to mapping scores. Edges with scores less than 0.1 are not shown. Nodes are colored by inferred Leiden community. **(D)** Expression of select "pan-invasive" genes in trophoblast of all species, grouped by SAMap homology group. Phenotypic covariates (endoreduplication, invasion, fusion) are annotated below cell type labels. Invasiveness groups by community, whereas fusion and endoreduplication are widespread. Md: *Monodelphis domestica*; Te: *Tenrec ecaudatus*; Cp: *Cavia porcellus*; Mm: *Mus musculus*; Mf: *Macaca fascicularis;* Hs: *Homo sapiens.*

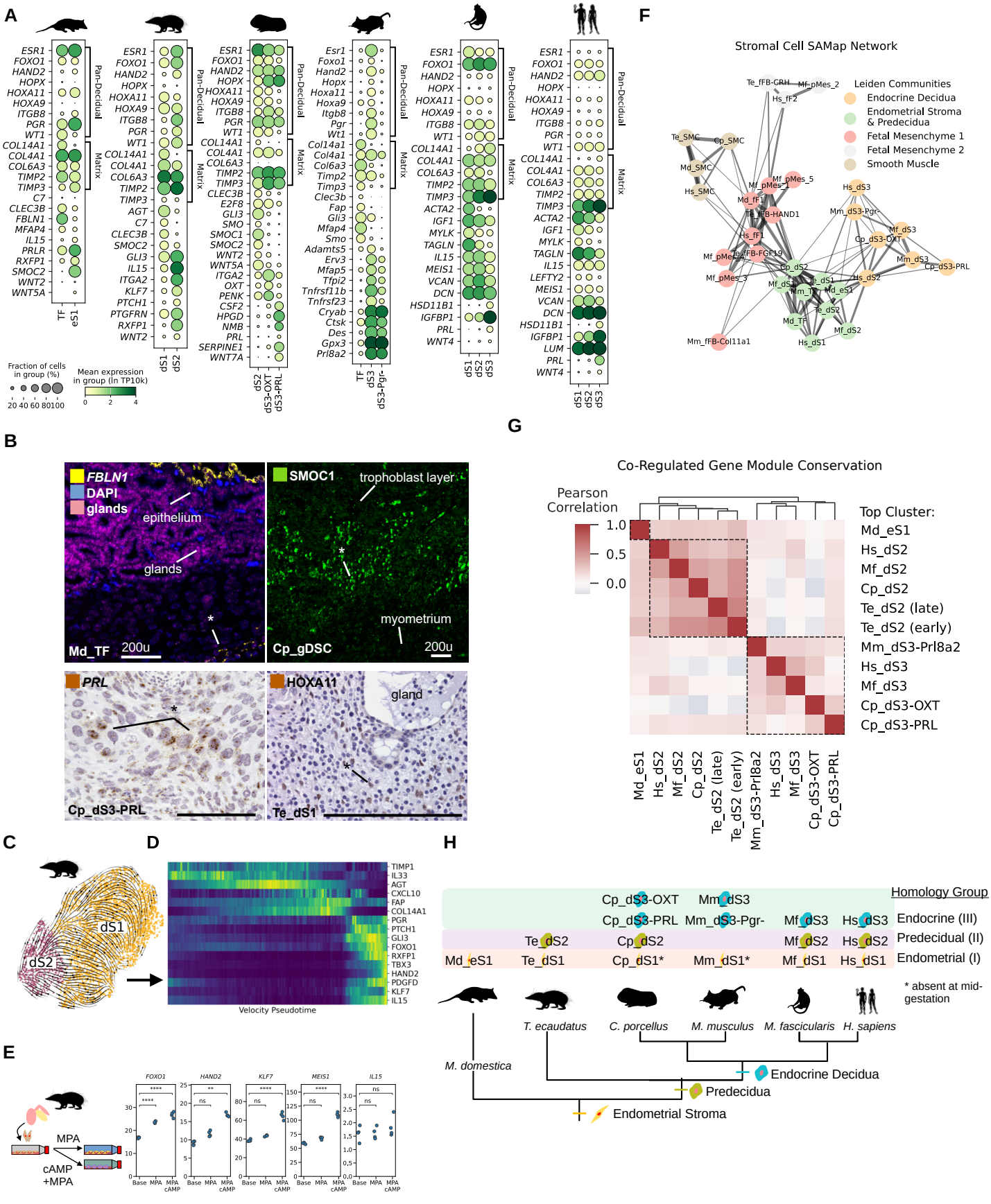

Figure 3. Cross-species comparison of decidual stroma. **(A)** Marker gene expression of endometrial stromal and decidual cell types. **(B)** *In situ* and immunohistochemical (upright) localization of select markers genes from **(A)**; cells of interest are marked by an asterisk (*). **(C)** Force atlas embedding of tenrec endometrial stromal cells with RNA velocity vectors overlaid. **(D)** Expression of select velocity-associated genes in the dS1→dS2 transition in all cells from the left plot, ordered by increasing velocity-informed pseudotime. **(E)** Expression of predecidual markers in primary tenrec uterine stromal cells cultured in vitro with deciduogenic stimuli (MPA: medroxyprogesterone acetate; cAMP) **(F)** Network of SAMap mapping scores between stromal cell types, with edge weight proportional to mapping scores, colored by community as identified by leiden clustering of the graph. Edges with scores less than 0.1 are not shown. **(G)** Correlation heatmap of NMF gene expression programs expressed in decidual stromal cells, with hierarchically-clustered dendrogram on top showing division into two families. **(H)** Tree showing inferred evolutionary relationships of decidual cell types.

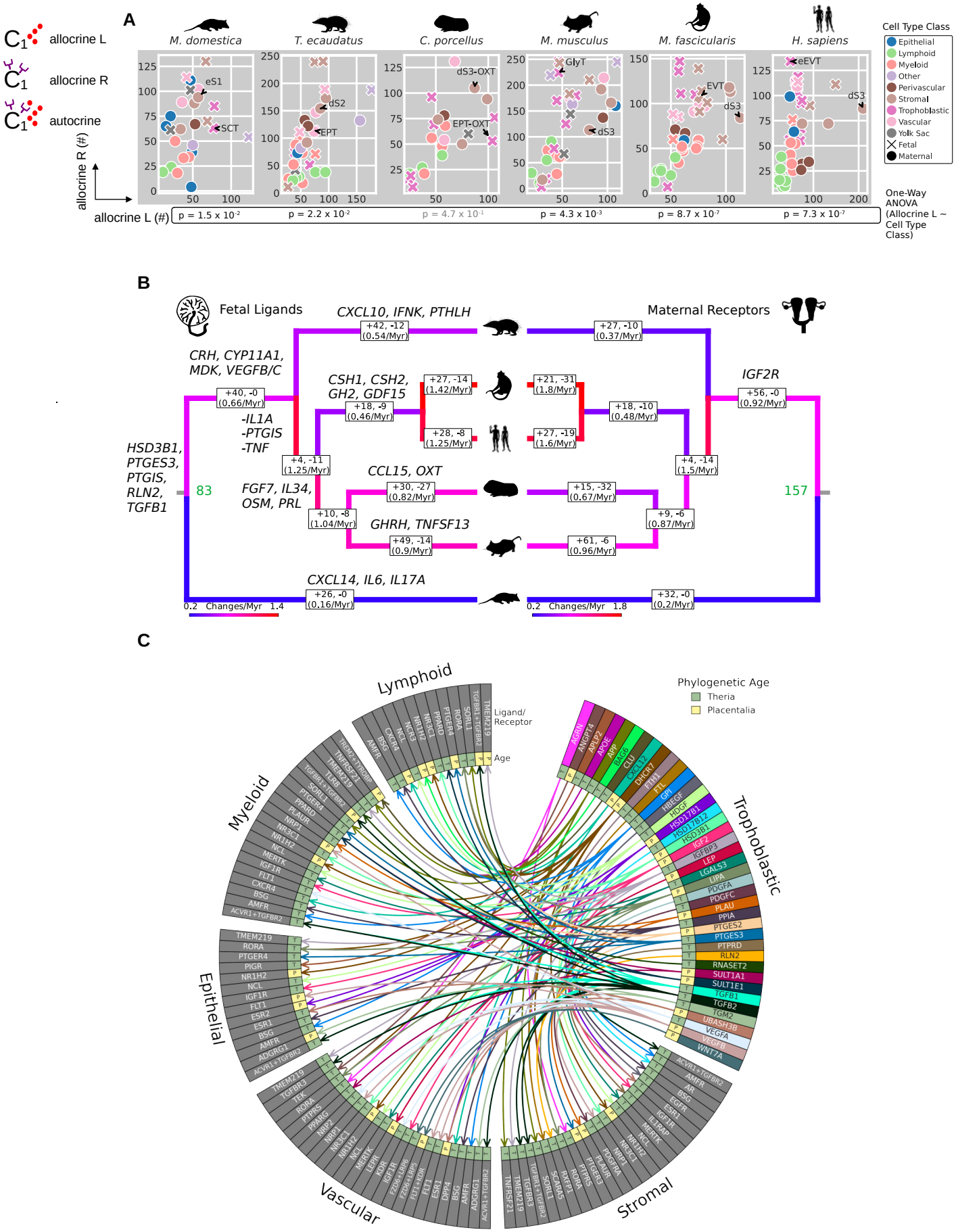

Figure. 4. Integration of cell types into cell-cell communication networks and reconstruction of evolutionary changes. (**A**) Numbers of allocrine ligands (receptor unexpressed) and allocrine receptors (ligand unexpressed) for secreted peptide signals. Degrees of integration range from low to high and stratify by cell type class. P-values of analysis of variance relating allocrine ligand degrees to cell type class in each species are plotted below. (**B**) Co-evolution of binary expression of signals by placental cells (left tree) and cognate maternal receptors (right tree) inferred from maximum-parsimony reconstruction. Branches are colored by rate (changes/Myr) using divergence dates from (56). (**C**) Reconstructed secreted signaling interactions in the Placentalia common ancestor, colored by phylogenetic age of gene expression (eutherian novelty: P; ancestral therian: T), partitioned by cell type class and subset to only those derived from the trophoblast.

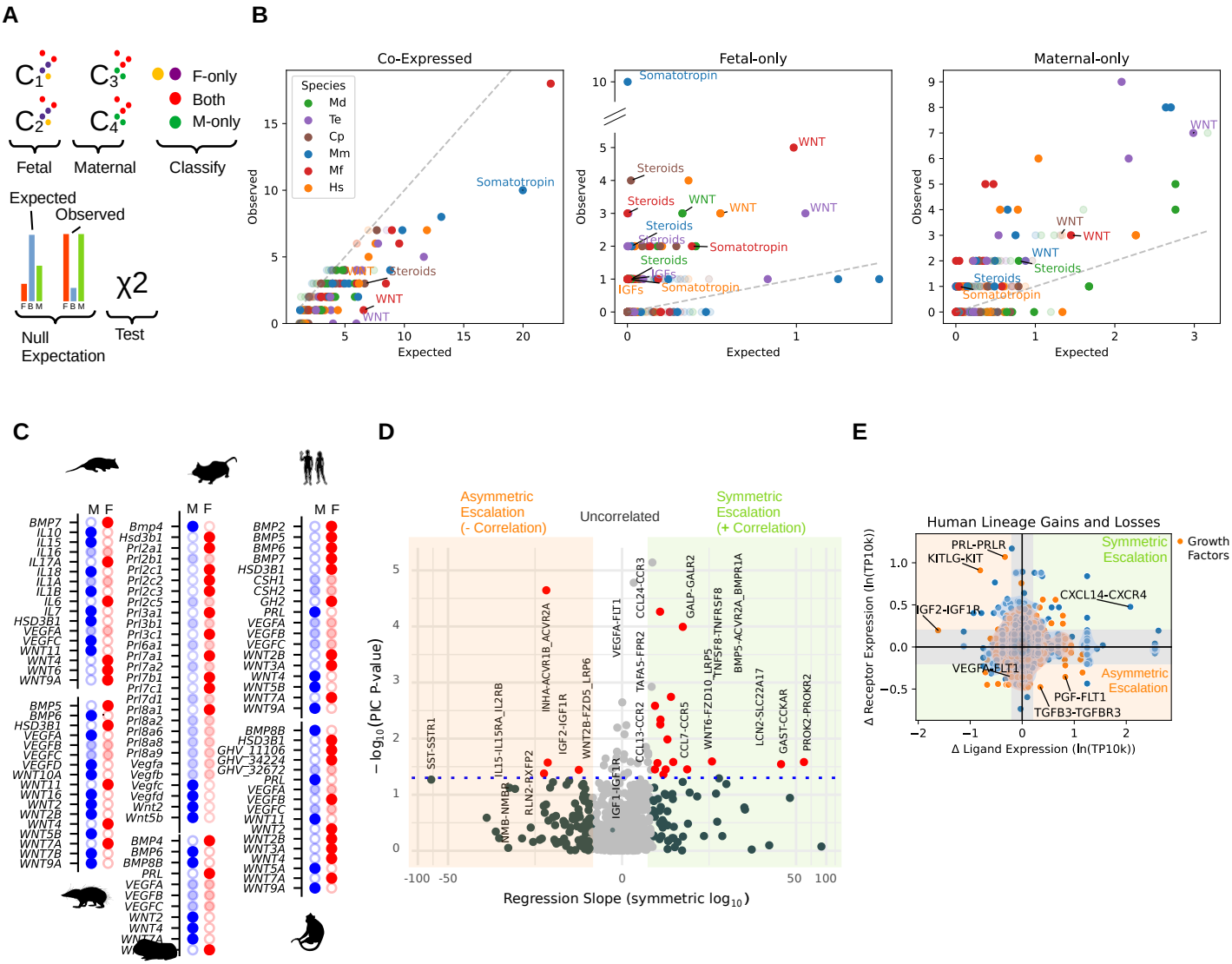

Figure. 5. Tests of two evolutionary hypotheses for evolutionary dynamics of fetal-maternal communication, “disambiguation” (8) (**A** to **C**) and “escalation” (9) (**D** to **E**). (**A**) Design for disambiguation test. (**B**) Observed vs. expected numbers of coexpressed, fetal-only, and maternal-only ligands, by ligand family. Points with solid color have both $p < 0.05$ and Benjamini-Hochberg false discovery rate $q < 0.05$. (**C**) Disambiguation status of select ligands. Blue: maternal-only; Red: fetal-only; faint: coexpressed. (**D**) Volcano plot of ligand-receptor pairs showing significant phylogenetically-independent correlation in expression (59) on the vertical axis and correlation slope on the horizontal axis. (**E**) Inferred evolutionary changes to ligand and corresponding receptor expression in humans with respect to the human-macaque common ancestor (Catarrhini).

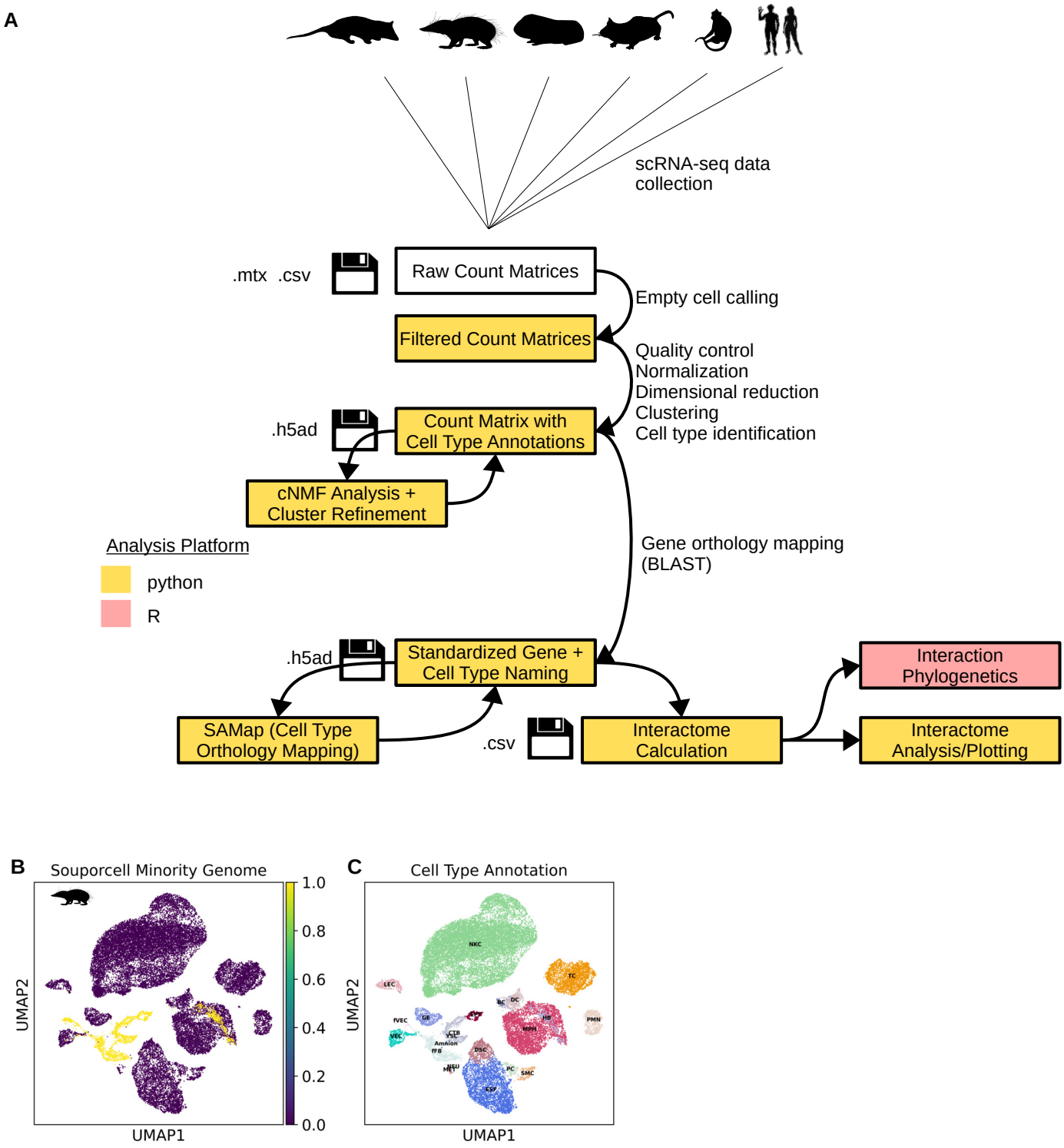

Figure S1. Single cell RNA-seq analysis. (**A**) Flow chart of analytical design. (**B** and **C**) Souporcell calls for *Tenrec ecaudatus* (k=2 genomes). UMAP embeddings of all cells are shown, colored by cell subtype on the left, and by whether or not the cell was assigned to the less frequent genome (assumed fetal) by souporcell on the right.

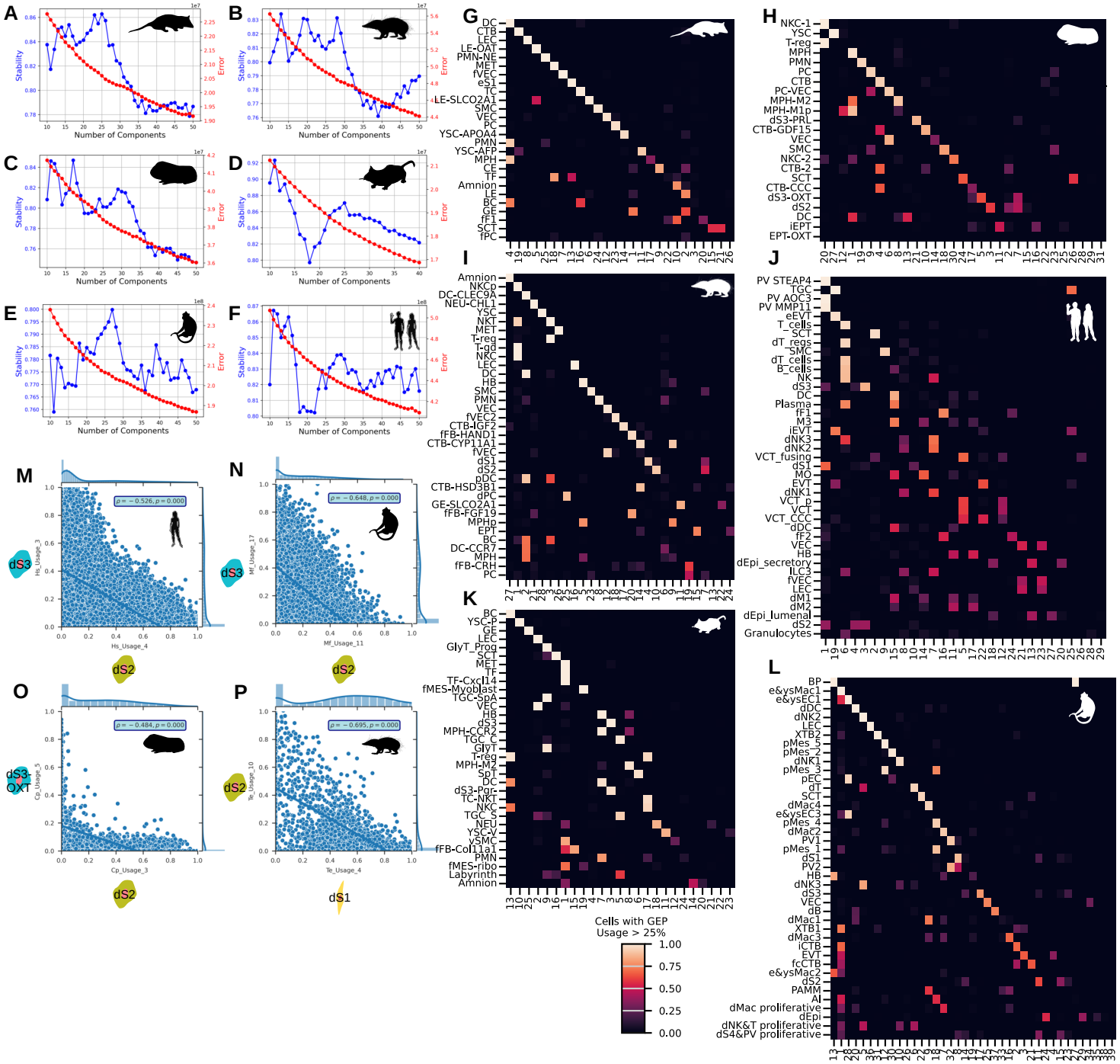

Figure S2. NMF analysis. (**A** to **F**) Optimal numbers of gene expression programs (NMF factors) were determined by identifying local stability values preceding a sudden drop. *Monodelphis domestica*: K=26; *Tenrec ecaudatus*: K=28; *Cavia porcellus*: K=31; *Mus musculus*: K=25. *Homo sapiens:* K=29. *Macaca fascicularis:* K=39. (**G** to **L**) Confusion matrices showing the mean activity score (ranging from 0 to 1) of cells in each cluster, taken from all cells with scores greater than 0.25. (**M** to **P**) Representative “Type II” and “Type III” decidual gene expression program activity scores across individual stromal cells of human (**M**), macaque (**N**), and guinea pig (**O**) show a diversity from exclusively Type II or Type III to a mixture of both programs. An equivalent comparison of Type I and II in the tenrec is depicted in (**P**).

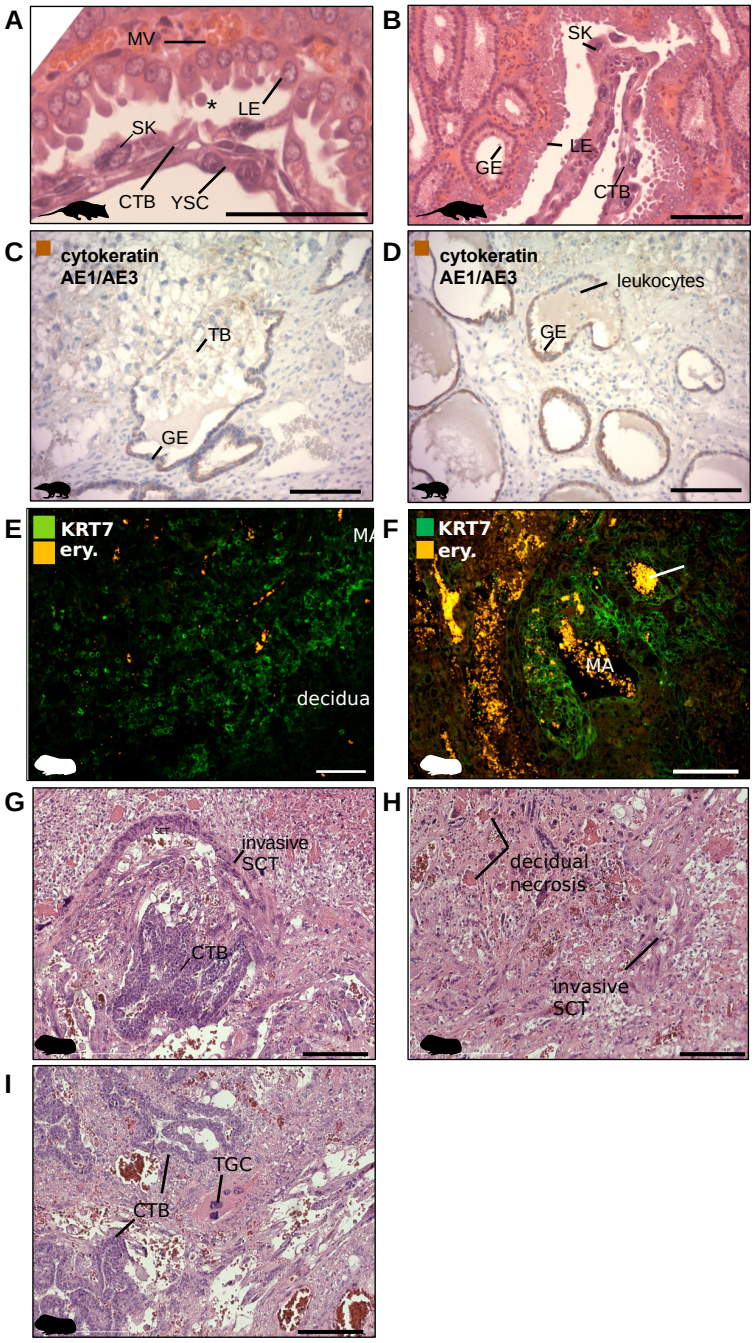

Figure S3. Additional histological identification of placental cell types. (**A** and **B**) The opossum fetal-maternal interface, showing 3 layers of yolk sac endoderm (YSC), cytotrophoblast (CTB), and syncytial knots (SK), in apposition with maternal luminal epithelium (LE) which produces secretory bodies (*); maternal glandular epithelium (GE) lies closely underneath the lumen. (**C** and **D**) The tenrec invasion front, where trophoblast invades into mucus-filled glands, eroding the glandular epithelium (GE) immunoreactive for pan-cytokeratin stain (CK AE1/AE3). (**E** and **F**) KRT7^{+} trophoblast in the guinea pig surrounding decidual stroma (left) and maternal arteries (MA; right). (**G** and **H**) Invasive syncytiotrophoblast (SCT) in the guinea pig emanates from subplacental cytotrophoblast (left) and extends into stroma where necrotic patches develop (right). (**I**) Trophoblast giant cell observed in the subplacenta of the guinea pig (proximate to subplacental CTB), but these cells were not captured by droplet-based sequencing. Scale bars: 200 µm.

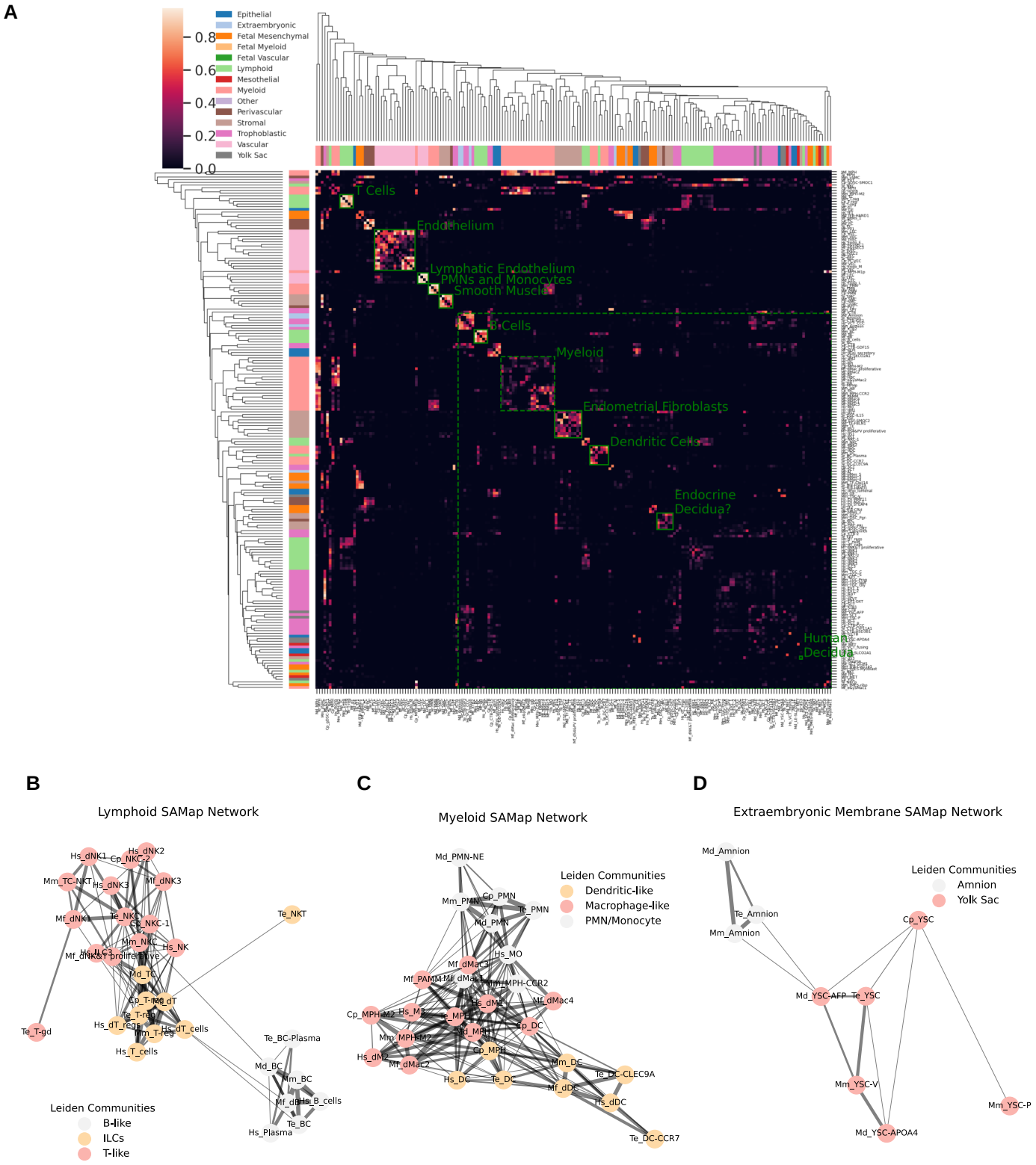

Figure S4. Cell type cross-species mapping scores (SAMap). (**A**) Clustered heatmap of pairwise cell type mapping scores (0-1), with selected 1:1 orthogroups annotated in green. (**B** to **E**) Networks subset from the full SAMap mapping table of (**A**) for specific cell type families, colored by Leiden community after community detection was run on the subnetwork. Nodes are cell clusters, and edge weights are equal to score magnitude, with scores less than 0.10 not shown.

A

Figure. S5. Cell type dendrogram of the cell types studied. (**A**) Neighbor-joining tree of transcription factor-only transcriptomes from all species studied.

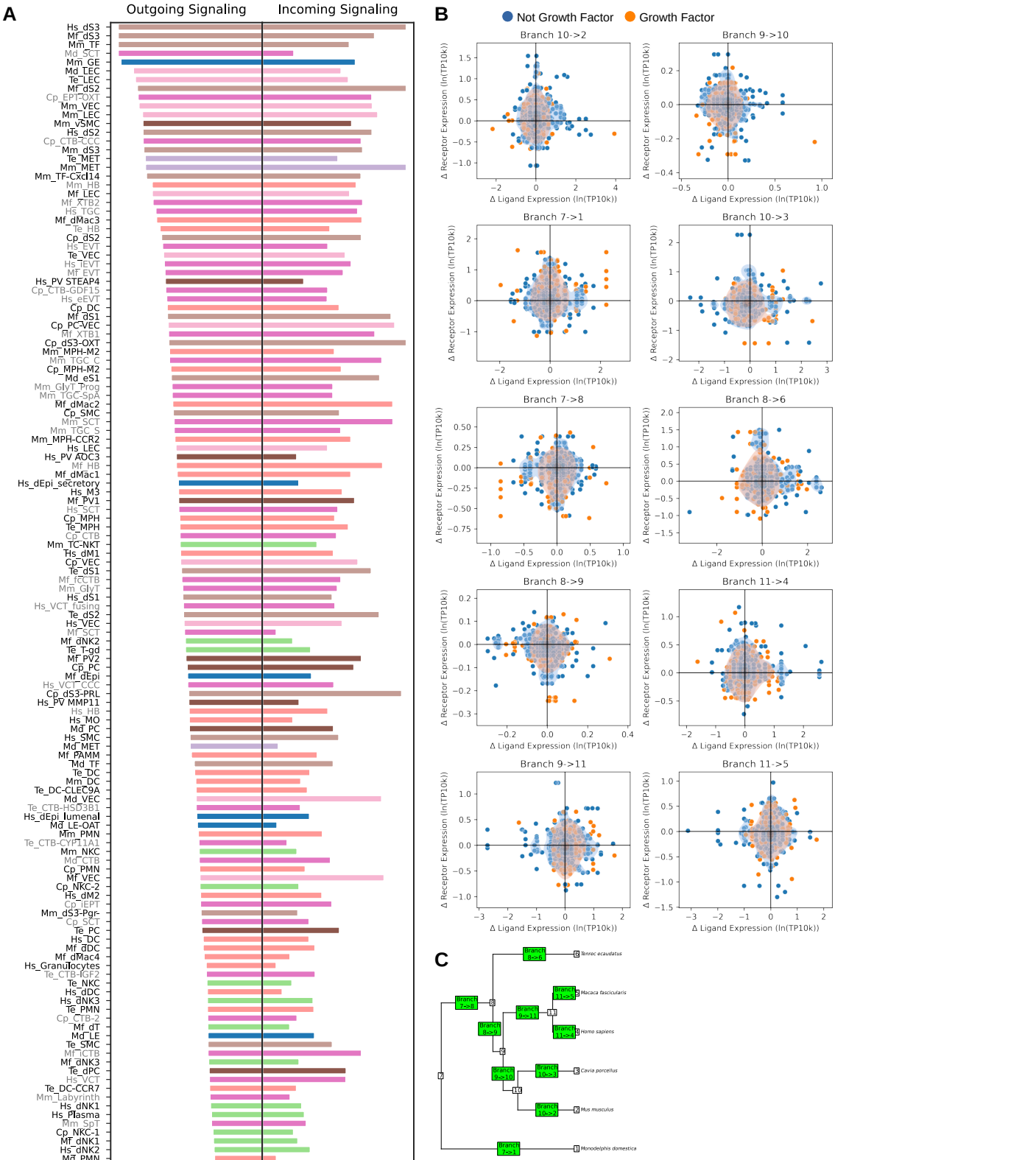

Figure S6. Extended cell-cell communication analysis. (**A**) Graph statistics of all cell types within their respective species' signaling networks. Kleinberg hub scores (a measure of outgoing signaling) are shown on the left and Kleinberg authority scores (a measure of incoming signaling) on the right. Graphs are weighted based on the product of expression values of ligand and receptor. (**B**) Inferred changes in inferred expression magnitude of placental ligands and cognate maternal receptors of secreted signals along internal branches of the phylogeny. Asymmetric escalation and de-escalation events fall into the lower-right and upper-left quadrants, respectively, and uncorrelated changes cluster around the center. (**C**) Phylogenetic tree of species sampled with numbering of nodes and branches labeled as in (**A**).

Hallmarks of uterine receptivity predate placental mammals

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Abstract

Embryo implantation requires tightly coordinated signaling between the blastocyst and the endometrium, and is crucial for the establishment of a uteroplacental unit that persists until term in eutherian mammals. In contrast, marsupials, with a unique life cycle and short gestation, make only brief fetal-maternal contact and lack implantation. To better understand the evolutionary link between eutherian implantation and its ancestral equivalent in marsupials, we compare single-cell transcriptomes from the receptive and non-receptive endometrium of the mouse and guinea pig with that of the opossum, a marsupial. We identify substantial differences between rodent peri-implantation endometrium and opossum placental attachment, including differences in the diversity and abundance of stromal and epithelial cells which parallel the difference between histotrophic and hemotrophic provisioning strategies. We also identify a window of conserved epithelial gene expression between the opossum shelled blastocyst stage and rodent peri-implantation, including *IHH* and *LIF*. We find strong conservation of blastocyst proteases, steroid synthetases, Wnt and BMP signals between eutherians and the opossum despite its lack of implantation. Finally, we show that the signaling repertoire of the maternal uterine epithelium during implantation displays substantial overlap with that of the post-implantation placental trophoblast, suggesting that the fetal trophoblast can compensate for the loss of endometrial epithelium in eutherian invasive placentation. Together, our results suggest that eutherian implantation primarily involved the re-wiring of maternal signaling networks, some of which were already present in the therian ancestor, and points towards an essential role of maternal innovations in the evolution of invasive placentation.

Introduction

Embryo implantation constitutes the first direct fetal-maternal encounter. It involves the blastocyst's apposition, adhesion, and - in species with invasive placentation - invasion of the blastocyst into the endometrium (Schlafke & Enders, 1975). In contrast to other forms of fetal-maternal contact, implantation involves breaching to some degree the maternal luminal epithelium by the embryo. Adhesion of the trophectoderm to the luminal epithelium requires modifications in cellular polarity on both sides (Denker, 1993). Disrupting endometrial integrity during the invasion by a semi-allogeneic embryo, on the other hand, requires taming of the inflammatory reaction (Griffith et al., 2017), a wound healing response (Nancy et al., 2018; Osokine et al., 2022), as well as avoiding immune rejection of the embryo (Medawar, 1953). Maternal innovations to overcome these challenges were necessary for implantation to evolve (Stadtmauer & Wagner, 2020a; Wagner, forthcoming).

Reconstructing the evolutionary origin of the sequence of developmental events involved in embryo implantation is difficult because of the lack of intermediate phenotypes. Invasive implantation likely evolved in the stem lineage of eutherian mammals (Wildman et al., 2006), coincident with invasive placentation and with the origin of the decidual reaction and the decidual stromal cell type (Mess & Carter, 2006). The type of epithelial penetration, the degree of invasion, and the orientation of the blastocyst upon attachment vary significantly even across closely related eutherian species (Siriwardena & Boroviak, 2022).

Implantation success depends on both the competency of the blastocyst and the receptivity of the endometrium. Success is determined by blastocyst chromosomal integrity and by maternal viability checkpoints (reviewed in Muter et al., 2023). It also depends upon endometrial remodeling of the epithelium and stroma, immune cell recruitment, vascular growth, and development of secretory endometrial glands. While several of these processes occur as part of the endometrial cycle regardless of fertilization, others are triggered and enhanced by the presence of the embryo. Endometrial

remodeling unique to pregnant cycles is known as “endometrial recognition of pregnancy” (Renfree, 2000), as opposed to “endocrine recognition of pregnancy”, which refers to serum levels of progesterone and estradiol differing significantly between pregnant and estrous cycles (Harder and Fleming, 1981). The latter is a derived characteristic of eutherian pregnancy. The blastocyst stage displays substantial morphological and developmental differences across mammals (Frankenberg et al., 2016), but also striking transcriptomic similarities at the cell type level of the embryo (Malkowska et al., 2022). However, the degree of evolutionary conservation in signaling between the blastocyst and the mother has remained unknown.

Much of what we know about eutherian implantation comes from mouse models, including genetically modified knockout mouse lines and their reproductive phenotypes (Dey et al., 2004, Wang & Dey, 2006). Aspects of implantation are variable across eutherians, and relying on single species thus confounds the species-specific characteristics with those of the larger group. In order to better represent rodents, we can look to a representative of the most basally branching rodent group, the guinea pig, with a long estrus cycle of around 16 days and an interstitial implantation phenotype resembling the human and differing from eccentric implantation of the mouse (Lee & DeMayo, 2004; Carter, 2007). The two species differ in the hormonal priming of the endometrium, with mouse pregnancy requiring a peri-implantation estrogen peak but not the guinea pig (Deanesly, 1960).

In contrast to rodents and other eutherian mammals, the gestation of most marsupials is characteristically short. However, marsupial pregnancy includes a stage shortly before parturition hypothesized to be homologous to the one in which implantation occurs in eutherians (Hughes, 1974; Harder et al., 1993; Griffith et al., 2017). At this stage, the shell coat surrounding the embryo has been dissolved and cellular contact between the trophoblast and endometrium is established. Following shell coat hatching, the uterine epithelium transforms in a way resembling the peri-implantation epithelial response in eutherians (Laird et al., 2014), although this transformation appears to primarily function in histotrophy rather than attachment (Griffith et al., 2019; Stadtmauer, Basanta et al., 2024). The opossum *Monodelphis domestica* has a gestation of 14.5 days from copulation to parturition (Mate et al., 1994), during the first 11 of which the fetus is surrounded by a proteinaceous shell coat and is fed by maternal uterine secretions (Zeller & Freyer, 2001).

We used single-cell transcriptomics to characterize the endometrial changes at early implantation in two rodent species, the mouse and guinea pig, in comparison with two time points during the short gestation of the marsupial *Monodelphis domestica*, which lacks implantation. In all three species, we targeted the stage when the pre-implantation embryo is a blastocyst, and maternal serum progesterone is elevated: 4.5 dpc for the mouse (Virgo and Bellward, 1974), 6.5 dpc for the guinea pig (Challis et al., 1971), and two stages of the opossum: 7.5 dpc, when the fetus is still a blastocyst (equivalent to the pre-implantation blastocyst in rodents) surrounded by the soft shell coat (Hinds et al., 1992; Yoshida et al., 2019), and day 13.5 of gestation, when the shell coat surrounding the fetus is lost and inflammatory attachment has begun **(Figure 1)**. To identify implantation-specific changes, we also sequenced the non-pregnant uterus from mouse diestrus and guinea pig luteal phase - cycle stages also characterized by high progesterone - and the non-pregnant, non-cycling opossum endometrium. We test for a conserved cellular signature of endometrial receptivity in eutherian species, and look for its equivalent in the opossum, and find that the opossum uterus already presents some of the hallmarks of eutherian implantation at the blastocyst-stage. We analyze time-matched preimplantation blastocysts in these species and find that gene expression and signaling potential of the fetal trophectoderm shows striking evolutionary conservation despite the considerable divergence of the eutherian implantation mode. Lastly, we compare signaling gene expression of the peri-implantation uterine epithelium to the trophoblast of the fully developed placenta, finding considerable overlap which may contribute to tissue stability after loss of the luminal epithelium in invasive placentation. Together, we characterize conserved and divergent characteristics of mammalian implantation and

identify possible constraints to the evolution of implantation due to the disruption of maternal tissue integrity during eutherian placentation.

Results

We generated single-cell sequencing libraries from the non-pregnant and peri-implantation endometrium of mouse, guinea pig and opossum **(Figure 2a-b)**, and generated cross-species integrated **(Figure 2c)** and species-specific **(Figure 2d)** two-dimensional embeddings using Uniform Manifold Approximation and Projection (UMAP). We annotated clusters into putative cell types using marker gene identification and refined annotations using the results of non-negative matrix factorization (cNMF) **(Figure S1)** and SAMap homology inference **(Figure S2)**.

Cell type inventories were highly similar between the rodents and opossum **(Figure 2d)**. In all three species, the non-pregnant uterus is predominantly composed of fibroblasts and smooth muscle cells, which make up more than 60% of captured cells **(Figure 2b)**. Relative cell type abundance diverged, however, at the peri-implantation stage. In our rodent species, uterine epithelial cells make up less than 10% of all cells, whereas in the opossum, more than 50% of all cells captured are epithelial at days 7.5 and 13.5 **(Figure 2b)**. This coincides with a pronounced expansion of the uterine glands in the second half of opossum gestation (Harder et al., 1993).

The opossum epithelium expresses some hallmark receptivity genes at the blastocyst stage but differs in expression dynamics

We first explore the similarity between rodent and opossum epithelial cell type composition and gene expression **(Figure 3a, see also Figure S2).** The mouse luminal epithelium displays the distinctive expression of *Ihh*, *Wnt7a*, *Wnt7b* and *Lrg5* (Seishima et al., 2019), as well high levels of *Tacstd2*, epithelial splicing regulatory proteins *Espr1* and *Espr2* (Hayakawa et al., 2017), and the transcription factors *Ehf* (Luk et al., 2018), *Msx1*, and *Klf5* **(Figure 3b)**. The guinea pig luminal epithelial cells express many of the same markers with the exception of *TACSTD2* **(Figure 3b)**. Opossum luminal epithelium includes one cluster enriched for *OAT* (Ornithine Aminotransferase, LE-OAT) and another enriched for *SLCO2A1*+ and *PTGS2*+ (Stadtmauer, Basanta et al., 2024). *OAT*+ luminal epithelial cells were transcriptomically more similar to mouse and guinea pig luminal epithelium (SAMap score of around 0.6) than *SLCO2A*1+ cells **(Figure 3a)**. In the opossum luminal epithelium, the expression of *IHH*, *LGR5*, *LIF* and *MSX2* was only detectable at the blastocyst stage (**Figure 3b**).

Glandular epithelial gene expression is well conserved across species and across stages (**Figure 3a; Figure S2**; SAMap score ≥ 0.8 in all pairwise combinations). Mouse glandular epithelium is characterized by the expression of *Foxa2*, serine proteases *Prss28* and *Prss29* (Dhakal & Spencer, 2021), *Spink1*, *Guca2b*, *Ltf* and *Sprr2f*. Conserved genes for the glandular epithelium across mouse and guinea pig included *FOXA2*, *ELAPOR1*, *PAX8*, *WWC1*, *MSX1, MSX2*, *WFDC2*, *EHF*, *KLF5*, *ESRP1/2* and *ST14* **(Figure 3b).** The opossum's glandular epithelium is also highly similar to that of the rodents, sharing the expression of *ELAPOR1*, *PAX8*, *WWC1*, *MSX1*, *MSX2*, *SOX9*, *PROM1*, *EPCAM, WWC1* and ST*14* **(Figure 3b).** *FOXA2* expression was higher in glands at the opossum blastocyst stage. Ciliated epithelial cells (CE) were also identified in the opossum, expressing markers of human ciliated cells (*FOXJ1* and *ADGB*; Garcia-Alonso et al., 2021), and with greatest SAMap affinity to glandular cells in the mouse and guinea pig (**Figure 3a**) even though there are no glandular ciliated cells in either species.

In the opossum, the abundance of ciliated cells (CE) and a specific luminal epithelial subpopulation (LE-SLCO2A1) increases towards mid-gestation. *OAT*$^{+}$ cells were enriched in the non-pregnant and day 7.5 stages, and *SLCO2A1*$^{+}$ cells were enriched on day 13.5. However, the most drastic change involves the increased abundance of glandular epithelial cells **(Figure 3c).**

In the mouse glandular epithelium *Foxa2*, *Prss28*, *Prss29*, *Guca2b* and *Spink1* showed higher expression at peri-implantation relative to diestrus, whereas *Lft*, *Sprr2f*, and *Spink12* showed decreased expression at peri-implantation **(Figure 3d)**. *Sprr2f* responds to circulating estrogen levels during the mouse's estrus cycle (Contreras et al., 2010), and it is up-regulated in the uteri of mouse with uterine-specific deletion of *Msx* genes (Sun et al., 2016). *Ltf* is a uterine epithelial-secreted protein also regulated by estradiol, required for both epithelial and stromal *Esr1* expression (Furuminato et al., 2023). Together, these expression dynamics indicate that uterine glands shift from an estrogen- to progesterone-dominated gene expression pattern at the time of implantation.

Genes upregulated in the opossum glandular epithelium at blastocyst-stage relative to non-pregnant, non-cycling stage showed substantial overlap with gene sets generated from experimental perturbations on cell lines and animal models available in the NCBI GEO repository **(see Methods)**. These included categories such as "estradiol mouse BAL cells" (GDS2562), "17beta-estradiol mouse uterus" (GDS1058), and "estradiol mouse uterus" (GSE23241) **(Figure 3e)**. The upregulation of estrogen-responsive genes in the glands at the opossum blastocyst stage constitutes a key difference with respect to the expression dynamics of mouse implantation, which shows an estradiol-progesterone shift. In addition, these results are consistent with the hypothesis that the suppression of estrogen signaling during implantation is a derived attribute of eutherian reproduction (Marinić et al., 2021).

In contrast, genes upregulated in the uterine glands of opossum at the 13.5 dpc placentation stage were enriched for genes stimulated by interleukin-1 (GDS2472; GDS4595), interleukin-15 (GSE59185), interleukin-10 (GSE59148), and interleukin-17A (GDS4601) **(Figure 3e).** This suggests that the inflammatory signaling in opossum late gestation also acts on the glands, consistent with a modulatory effect of inflammation on histotrophic activity of the mother.

Rodent stroma shows greater cell heterogeneity and proliferation at peri-implantation

Mouse fibroblasts separated into three transcriptomically distinct populations associated with different histological microenvironments: subepithelial, inner stromal, and myometrial tissue fibroblasts **(Figure 4a)**, consistent with previous studies of peri-implantation mouse endometrium (Kirkwood et al., 2021). All three populations express *Pdgfra*, *Hoxa9, Hoxa10, Hoxa11*, and *Pgr*. We identified subepithelial fibroblasts (ESF_subepi) by their enriched expression of *Angptl7*, *Aspg*, and *Ptch2*. Fibroblasts in the inner stroma (ESF_inner) are characterized by enriched expression of *Hand2*, whereas fibroblasts close to the myometrium (TF), also referred to as "tissue fibroblasts", are enriched for *Fbln1*, *Mmp3*, *Lum*, *Ecm1*, *Clec3b*, and *Fap* **(Figure 4b)**.

Guinea pig endometrial fibroblasts clustered into three sub-populations as in the mouse, with high SAMap scores between the corresponding clusters of the two species **(Figure 4a, see Figure S2)**. All endometrial stromal fibroblasts share expression of *PDGFD* and its receptor *PDGFRA*, *HOXA11*, and *SERPINF1*. Tissue fibroblasts express *LUM*, *FBLN1*, *ECM1*, *CLEC11A*, and *DPT* **(Figure 4b)**.

Opossum fibroblasts segregated into only two cell populations, *SMOC2*-positive endometrial stromal (ESF-SMOC2) and *FBLN1*-positive tissue fibroblasts (TF-FBLN1) (Stadtmauer, Basanta et al., 2024). Eutherian endometrial stromal fibroblasts respond to hormonal changes and differentiate into decidual cells. The opossum endometrial stromal fibroblasts show high similarity to eutherian

endometrial fibroblasts **(Figure 4a)**, both clusters sharing the expression of *HOXA11* and *PGR*, despite the inability of the opossum's stroma to decidualize (Kin et al., 2014; Erkenbrack et al., 2018). A notable difference between rodent stromal fibroblasts and the opossums was the lack of significant levels of *HAND2* expression, as well as lower levels of *HOXA* transcription factors **(Figure 4b)**.

Mouse and guinea pig endometrial inner stromal fibroblasts include a subpopulation characterized by numerous proliferation markers. These proliferating stromal cells increased in abundance at the rodent peri-implantation stages **(Figure 4c)** and were absent in the mid and late-gestation opossum endometrium. Finally, there were no substantial differences between the stromal fibroblast gene expression of opossums during mid and late-gestation stages, besides a precipitous decline in abundance **(Figure 4d)**, suggesting that stromal transformation is not involved in attachment or endometrial recognition of pregnancy in the opossum as it is in deciduate (placental) mammals.

Main eutherian implantation signals are present in the opossum epithelial-stromal crosstalk before egg-shell rupture

We inferred maternal cell communication potential from cell type transcriptomes **(see Methods; Figure S3)** and assessed putative cell-cell communication at the epithelial-stromal crosstalk. Then, we explored differential signaling from epithelium to the stroma between the peri-implantation phase and non-pregnant stages in rodents, as well as between days 7.5 and 13.4 and non-pregnant stage in the opossum **(Figure 5)**.

In mouse and guinea pig luminal uterine epithelium, *IHH* was inferred to signal to stromal fibroblasts **(Figure 6a-b)**. In the mouse, *Ihh* signaling was not significantly upregulated at peri-implantation compared to diestrus but the Ihh-receptor complexes *Boc_Ptch1* (2.78-fold, $p = 1.6 \times 10^{-9}$) and *Cdon_Ptch1* (2.64-fold, $p = 4.2 \times 10^{-3}$) were **(Table S1.1)**. In the guinea pig, we found the opposite trend: *IHH* displayed a 2.41-fold upregulation of the ligand at peri-implantation relative to diestrus ($p = 0.06$), but not its receptor complexes *BOC_PTCH1* ($p > 0.1$) or *CDON_PTCH1* ($p > 0.1$) **(Table S1.2)**.

IHH was detected in the glandular epithelium of the opossum at 7.5 dpc **(Figure 6c)**, showing a significant increase from the non-pregnant, non-cycling state to 7.5 dpc (7.11-fold, $p = 8.67 \times 10^{-7}$) **(Table S1.3)**. In the placentation phase at 13.5 dpc, glandular *IHH* expression is reduced to near-zero, suggesting that its expression is limited to a narrow window around the blastocyst stage. With respect to the potential for epithelial-stromal *IHH* signaling in this species, *PTCH1* is highly expressed in opossum endometrial stromal fibroblasts (180.0 TPM), but its co-receptors *BOC* (no ortholog identified), *CDON* (4.6 TPM), and *GAS1* (2.0 TPM) are not, leading the receptor complex to be called as "off" in our cell communication inference analysis **(Figure 6c)**. However, given the expression of both the ligand and its main ligand-binding receptor subunit in opossum, we suggest that this active signaling pathway may be conserved across therians, although experimental verification will be necessary.

Leukemia inhibitory factor (*LIF*) signaling from glandular and to a lesser extent luminal epithelium was detected in the mouse, where *Lif* production showed nominal upregulation in epithelial cells on day 4.5 versus diestrus (1.87-fold increase, p= 0.35) **(Table S1.1).** The guinea pig, in contrast, lacked luminal or glandular epithelial *LIF* expression, and therefore signaling via this pathway was not inferred **(Figure 3b; Figure 6b)**. *LIF* expression can rescue implantation in mice under low-estradiol conditions (Chen et al., 2000), suggesting that it mediates estrogen signaling, a function that may not be needed in the guinea pig. In the opossum, *LIF* signaling from the luminal epithelium to the stroma was present at day 7.5, but reduced in magnitude (7.7-fold decrease, $p = 5.1 \times 10^{-6}$) compared to the expression in the non-pregnant stage and not expressed at day 13.5 **(Figure 6c-d)**. These data suggest

that LIF signaling to the stroma is not related to endometrial recognition of pregnancy in opossum **(Table S1.4).**

In mouse peri-implantation, we identified potential epithelial *Wnt7a*, *Wnt7b,* and *Wnt11* signaling from the epithelium to the stroma via frizzled receptors *Fzd1*, *Fzd2* and *Fzd3* **(Figure 6a)**. For the signaling involving *Wnt7b*, the receptor *Fzd1*, along with several secreted frizzled related proteins (SFRPs), were significantly upregulated during implantation compared to diestrus **(Table S1.1)**. In guinea pig peri-implantation, we identified *WNT7B* signaling from luminal epithelium to the glands **(Figure 6b)**. In the non-pregnant opossum, *WNT5A, WNT7A, WNT7B,* and *WNT11* were expressed (>20% of cells in the cluster) in luminal epithelium, but were downregulated below our expression threshold at day 7.5 (*WNT7A* $p = 3.68 \times 10^{-11}$; *WNT7B*: p=0.022; *WNT11*: $p = 1.38 \times 10^{-23}$ *WNT5A*: $p=6.96 \times 10^{-13}$) compared to the non-pregnant **(Table S1.3)**, and remained off at day 13.5 **(Table S1.4)**. In summary, rodent uterine epithelium expresses peri-implantation Wnt signaling which is absent in the opossum **(Figure 6c-d)**.

Blastocyst signaling is conserved across Theria

To complement uterine signaling, we assessed the putative signaling between the blastocyst and uterine epithelium in mouse, human, and opossum, utilizing published blastocyst expression data on opossum E7.5 bilaminar blastocyst (Mahadevaiah et al., 2020), mouse E4.5 (Nakamura et al., 2015), guinea pig E5.5 (Guan et al., 2024), and human day 7 (Petropoulos et al., 2016). In addition, we included data from the human endometrial mid-secretory epithelium (Marečková et al., 2024).

We first inferred and investigated the potential signaling between the trophoectoderm and the maternal epithelium. We found that in all four species, the trophectoderm shows potential to signal to the maternal epithelium by Wnt ligands. In the mouse, these include *WNT3A, WNT6, WNT7B,* and *WNT9A* **(Figure 7a)**. Human data suggests trophectoderm signaling to the luminal epithelium via *WNT3, WNT5B, WNT6, WNT7A,* and *WNT7B* **(Figure 7b)**. In the guinea pig, it included *WNT6* and *WNT3* **(Figure 7c)**. In opossum, blastocyst-expressed Wnt ligands with epithelial uterine receptors included *WNT3A, WNT6, and WNT11* **(Figure 7d)**. Interestingly, in the opossum, the Wnt signaling had concomitant receptors in the glandular instead of the luminal epithelium. The trophectoderm of the four species also expressed BMP family ligands, including *BMP8A* in the mouse, *BMP8A*, *BMP4* and *BMP2* in human, and *BMP4* and *BMP2* in the opossum **(Figure 7)**.

Signaling by *IL6*, a trophectoderm marker involved in signaling to the inner cell mass (Plana-Carmona et al., 2022), *LIF* (Cullinan et al., 1996) and *IGF2* (Rappolee et al., 1992) was not conserved: whereas *IL6* was inferred to signal to the luminal epithelium only in the human, *LIF* was detected only in the rodents, and *IGF2* only in mouse trophectoderm **(Figure 7a-c)**. Expression of growth differentiation genes such as *GDF11* was unique to eutherians.

We then explored conservation of blastocyst gene expression. Enzymes involved in steroid biosynthesis in the blastocyst also showed conservation across species, including dehydrogenase enzymes involved in cholesterol metabolism and estrogen synthesis such as *HSD17B7*, *HSD17B11* and *HSD17B12* (Luu-The et al., 2006; Prehn et al., 2009) **(Figure 8a)**. Blastocyst-secreted proteases function in implantation to digest the layers covering the embryo (Salamonsen & Nie, 2002; Denker & Tyndale-Biscoe, 1986; Selwood, 2000). Trophectoderm protease expression was found to be highly conserved across the marsupial, rodent, and primate species, including *ADAM* family genes and cathepsins **(Figure 8b)**. This suggests that protease expression in the extraembryonic membranes is conserved among therian mammals (including marsupials) at the blastocyst stage.

The conserved presence of protease expression in the trophectoderm from opossums to humans is thought to elicit a maternal response at implantation characterized by cellular stress (Brosens et al., 2014; Erkenbrack et al., 2018) and inflammation (Griffith et al., 2017). We investigated the degree to

which the uterus at peri-implantation appeared to respond to signaling from the embryo in this way. Day 7.5 of opossum gestation shows an increase in abundance of inflammatory immune cells (PMN) that is maintained at day 13.5 **(Figure 8c)**. The opossum inflammatory attachment reaction has been reported to be characterized by expression of *IL1A, IL6, IL10, IL17A, CXCL8,* and *TNF* (Griffith et al., 2017; Hansen et al., 2017). However, few of these were upregulated in our rodent samples at implantation, although the number of macrophages captured increased **(Figure 8d)**. However, differential gene expression analysis of macrophages between diestrus and peri-implantation in the mouse showed upregulation of genes such as *Clec4d*, *Il1r2*, *Il1rn*, *Cxcl1*, *Cxcl2* and *Lif,* suggesting a transition in macrophage polarization towards a pro-inflammatory profile **(Figure 8e)**. We have also previously reported that some inflammatory mediators are expressed by the 13.5 dpc opossum placenta are produced by the syncytial trophoblast **(Figure 8d)** (Stadtmauer & Wagner, 2020b; Chavan et al., 2021; Stadtmauer, Basanta et al., 2024). From these comparisons, it is unclear that trophectoderm expression of proteases at peri-implantation is associated with an inflammatory response. A study of post-attachment stages would be required to resolve this question with greater precision.

The post-implantation interface maintains signaling continuity by cell type substitution

Paracrine interactions between maternal cell types are critical for the establishment and maintenance of pregnancy. These interactions include the remodeling of endothelial cells and the regulation of immune cells. We traced the conservation of signaling between four major functional cell type classes - epithelial, stromal, macrophage, and endothelial cells - between mouse and guinea pig peri-implantation and the opossum 7.5 and 13.5 dpc stages, and how these interactions change later in pregnancy after the stroma and epithelium are remodeled.

Luminal epithelium in all species expressed high levels of vascular endothelial growth factor (*VEGF*) and placental growth factor (*PGF*), pro-angiogenic ligands for the endothelial cell receptor *FLT1* (Ribatti, 2008) **(Figure 9a; Table S2)**. Macrophages demonstrated signaling to endothelial cells via *IGF1* and *TNF* in mouse, guinea pig, and 7.5 dpc opossum (**Figure 9a**). Signaling from macrophages to stromal cells included *TNF* and *TGFB1* (**Figure 9a**). Signaling from the stroma to the epithelium included *BMP2* and *IGF1* in rodents and opossum **(Figure 9a; Table S2.3-4)**. Stromal-to-macrophage signaling included *CXCL12* and prostaglandin E2 via the synthase *PTGES3* in both rodents and the opossum **(Figure 9a; Table S2.1-2)**.

The development of the invasive fetal-maternal interface involves a major change in constituent cell types present in the endometrium: the luminal epithelium is eroded and replaced by the trophoblast, and endometrial stromal fibroblasts differentiate into decidual stromal cells **(Figure 9a)**. To understand how the roles of single cell types change in this tissue restructuring, we first compared the expression of secreted ligands between endometrial stromal fibroblasts at the rodent peri-implantation period to the decidual ligands of the established fetal-maternal interface (Stadtmauer, Basanta et al., 2024). Considerable divergence of secreted signaling was found between mid-gestation decidual cells and their developmental precursors in peri-implantation mouse (Jaccard similarity index = 0.36 in the range from 0-1) as well as in guinea pig (Jaccard index = 0.48 for PRL+ decidual cells, 0.53 for OXT+ decidual cells) **(Figure 9b; Table S3)**.

We predicted that the successful establishment of pregnancy, which involves a physical replacement of maternal luminal epithelium by the trophoblast in eutherian mammals, may require a continuity of epithelial signaling interactions. Using data from our previous study (Stadtmauer, Basanta et al., 2024), we calculated Pearson correlations between the secreted ligands repertoires of mid-gestation cell types with the uterine epithelium of blastocyst attachment stage mouse, guinea pig, and opossum. In all three species, integrated trophoblast cell types were among the most similar in

their secreted ligands to the uterine epithelium **(Figure 9c)**. In the mouse, invasive trophoblast giant cells (glycogen and spiral artery-remodeling) had greater epithelial similarity than non-invasive (canal and sinusoidal). In the guinea pig, subplacental cytotrophoblast showed greater similarity to uterine epithelial cells, and in the opossum, cytotrophoblast is most similar to uterine epithelium (**Figure 9c-d**).

We compared the overlapping ligands between the top-scoring placental cell types resulting from the Pearson correlation and the peri-attachment epithelium ligands **(Table S4)**. In all species, the set of ligands produced by invasive trophoblast was smaller than those produced by the peri-implantation epithelium. Among the non-overlap, epithelial-specific ligands in mouse included receptivity markers such as *IHH, WNT*, and *LIF* and trophoblast-specific ligands included *BMP8A* and *IGF2* **(Figure 10a)**. In the guinea pig, *IHH* and *WNT* are unique to the epithelium, *LEP* and *OXT* to the extraplacental trophoblast and *GDF15* to the subplacental cytotrophoblast **(Figure 10b)**. In the opossum, *BMP8A* and *IHH* were also cytotrophoblast and epithelium specific respectively. The syncytiotrophoblast expressed inflammatory mediators as mentioned above **(Figure 10c)**. In humans, the extravillous trophoblast expresses *LEP* and *IGF2* and the epithelium *IHH*, *WNT* and *LIF* **(Figure 10d)**. Because of the relationship between invasive trophoblast subtypes and vascular remodeling, we next tested what proportion of the overlapping ligands between the epithelium and the top-scoring trophoblasts subtypes is involved in vascular interactions, that is to say, which ligands have receptors in the vasculature. Among the overlapping ligands between epithelium and the top-scoring trophoblast match in rodents, a substantial portion of ligands had expressed receptors in vascular cell types of endothelial cells and pericytes. In the mouse, these included *ANGPT2*, *HDGF*, stem cell factor *KITLG*, *VEGFA*, *VEGFB*. In the guinea pig, the vascular growth factors *VEGFA, VEGFB, VEGFC, PENK, VWF* and stem cell factor *KITLG*. We found that in rodents a substantial percentage of the overlapping ligands had indeed receptors in the vasculature (51% and 40% in mouse and guinea pig respectively). A considerable percentage was also in the opossum, falling slightly behind the rodents with 37.5% of the shared ligands having concomitant receptors in the vasculature (**Figure 10e**). Overall, the pattern suggests that in rodents the trophoblast takes over part of the luminal epithelial signaling function, and that the ability to substitute for the epithelium was an ancestral feature of the therian trophoblast which may enable its loss in invasive placentation.

Discussion

Embryo implantation constitutes the most intimate and specialized cellular contact between the embryo and the mother. This first contact is essential in eutherians to establish the placenta and initiate stable gestational development. We compared two rodent species and examined the evolutionary conservation of the eutherian implantation uterine response in contrast with the opossum, which lacks implantation and a prolonged gestation period. This comparison reveals insights into the evolutionary origins of implantation.

Knowledge of expression patterns and signaling crosstalk during implantation largely derives from studies on mice (Hantak et al., 2014; Yang et al., 2021), with limited molecular information from other rodent species (Blandau, 1949; Enders & Schlafke, 1969; Cha & Dey, 2015). As mouse and guinea pig are phylogenetically distant, with the common ancestor being Rodentia's most recent common ancestor, including the guinea pig in our analysis helped to provide broader insight into rodent reproductive biology. The structure and cell biology of the placental interface established later in pregnancy differ substantially between mouse and guinea pig (Stadtmauer, Basanta et al., 2024). At the implantation stage, however, we found a broadly conserved implantation biology between the two species in cell-type composition, gene expression in individual cell types and cell-cell communication.

We generated single-cell atlas of the implantation and non-receptive endometrium of mouse and guinea pig, as well as the non-pregnant, mature blastocyst, and placental attachment stages of the gray short-tailed opossum *Monodelphis domestica*. This marsupial has been established as an outgroup species to eutherians and used to infer the reproductive changes that evolved with eutherian embryo implantation (Griffith et al., 2017). The endometrium of opossum consisted of largely homologous cell types to that of mouse and guinea pig, we observed greater epithelial cell heterogeneity in the opossum and greater stromal cell diversity in rodents, suggesting that the divide between hemotrophic and histotrophic biologies in marsupials and eutherians is represented at the level of cell type diversity. Further research will be required to determine whether the pregnancy-specific differentiation of the opossum luminal epithelium to a secretory state enriched in late pregnancy (LE-SLCO2A1), with lower SAMap mapping scores to mouse and guinea pig luminal epithelium, represents an evolutionarily novel cell type in marsupials. Likewise, opossum *FOXJ1*$^{+}$ ciliated epithelial cells (CE) had no equivalent in mouse and guinea pig, as in these species ciliated epithelium is restricted to the oviduct, unlike in humans, which also have ciliated uterine epithelial cells (Hunter et al., 2024). Further comparative research of oviductal epithelium from more species will be required to assess whether this cell type evolved independently in marsupials and eutherians.

The dynamics of cell type abundance in response to hormonal changes also differed vastly between marsupials and eutherians. In *Monodelphis domestica,* the subepithelial stroma undergoes remodeling during the first 7 days of pregnancy, wherein endometrial fibroblasts that are abundant in the non-pregnant uterus are replaced by uterine glands, which produce histotrophic nutrition for the offspring (Griffith et al., 2019). While glands predominate within the endometrium of the opossum after 7.5 dpc, the stroma dominates in abundance in rodents and shows a parallel regional differentiation into subepithelial, inner stromal, and myometrial populations in both mouse and guinea pig. Given the supportive role of uterine glands in marsupials, the shift from glands to the stroma as a major source of support for the embryo has likely been one of the essential steps in the evolution of eutherian implantation.

It is not straightforward to identify homologous stages between the opossum's short gestation and that of eutherians based on transcriptional similarity. Denker & Tyndale-Biscoe (1986) used "(superficial) implantation" to refer to the specialized cellular contact between extraembryonic membranes and endometrium which occurs before parturition in macropodid marsupials such as kangaroos and wallabies. In the opossum, this would apply to the day 12-13.5 of pregnancy. Furthermore, inflammatory cytokine expression during the last two days of opossum gestation has been proposed as homologous to inflammation during eutherian implantation (De Filippo et al., 2013; Hendriks et al., 2008; Griffith et al., 2017). For this reason, it is surprising that our comparative analysis shows greater similarity of rodent pre-implantation (mouse 4.5 dpc, guinea pig 6.5 dpc) to the blastocyst stage of opossum (day 7.5 dpc; blastocyst stage) than to placentation (13.5 dpc). We find that during this window, *IHH*, *WNT7A*, and *MSX2* are expressed, along with the glandular epithelial expression of *FOXA2* and the serine protease *ST14*. These timepoints share additional similarities in embryonic staging (summarized in Malkowska et al., 2022) and elevated circulating progesterone **(Figure 1)**. Indeed, some of these genes, such as *IHH*, are progesterone-responsive (Matsumoto et al., 2002), suggesting that this window of similarity may represent a uterine response to hormonal changes of early pregnancy. The 7.5 dpc stage of opossum pregnancy is also, like the implantation window of humans (Muter et al., 2023), the period when pregnancy failure is most likely to occur (Yoshida et al., 2019). Wnt signaling, on the other hand, was found to be expressed in non-cycling opossum endometrium but not at either pregnant timepoint. This suggests that prolonged epithelial Wnt signaling is likely a derived component of the eutherian implantation process. Together, these results demonstrate that the expression of some of the signaling pathways that were already functional in the

uterus of the last common ancestor of marsupials and eutherians were later recruited into eutherian implantation.

We found high conservation of gene expression between the blastocyst trophectoderm of the opossum, rodents, and the human, including proteases, steroid biosynthetic enzymes, and Wnt and BMP ligands with receptors expressed in the endometrium. Conservation suggests that the signaling potential of the blastocyst has not undergone dramatic change in therian evolution, and as a result we must attribute major changes underlying the innovation in implantation mode in eutherians to changes in the maternal tissue. Elucidation of precisely what these maternal innovations were is a deserving focus of future investigation. That said, one source of fetal evolutionary change despite strong conservation is neo-functionalization of ancestral trophectoderm products such as proteases, which presumably lost their functional necessity for hatching once the shell coat was evolutionarily lost in stem eutherians (Selwood, 2000). Serine proteases have been reported to function in matrix dissolution to aid placental invasion (Salamonsen & Nie, 2002) and in embryo screening (Brosens et al., 2014), two functions which likely diverge from their ancestral role.

Comparison of the secreted signaling repertoire of the uterine epithelium at the peri-implantation stage and the trophoblast at mid-gestation revealed continuity of interactions throughout pregnancy. Our results suggest that eutherian implantation is a transition in tissue composition, where the luminal epithelium is replaced by the trophoblast, forming a meta-stable tissue configuration: the maternal-fetal interface (Pavlicev & Wagner, 2024). The high similarity between the signaling repertoires of trophoblast and uterine epithelium indicates that the trophoblast is uniquely capable of mediating this transition. The opossum, which lacks decidualization and epithelial erosion during placentation, nevertheless showed overlap of cytotrophoblast secreted signaling with its uterine luminal epithelium, suggesting that this substitution capacity reflects a conserved epithelial nature of the therian trophoblast. Redundancy between trophectoderm and epithelial signaling may have allowed the transition to invasive forms of placentation to evolve in the stem eutherian lineage without greater disruption to endometrial signaling networks.

Altogether, our data indicate that hallmarks of the eutherian endometrial receptivity - specifically pre-implantation changes to the luminal epithelium - are shared between rodents and the opossum and thus predate the origin of invasive implantation in placental mammals. This suggests that necessary cell-biological prerequisites for eutherian invasive implantation may have existed in the therian common ancestor, prevented by the presence of the shell coat which prevents direct trophoblast-uterine contact until late gestation; thus, the loss of this membrane thus may have precipitated major changes in placentation and reproductive mode in the eutherian lineage. Further comparative research is needed to clarify this sequence of evolutionary events and to reconcile it with the hypothesis that post-hatching cell-cell contact is the marsupial homolog to the eutherian implantation stage.

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Materials and Methods

Animals and sample collection

C. porcellus (Charles River) were maintained at the University of Vienna according to Institutional Animal Care protocols. The estrus cycle was monitored by examination of vaginal membrane opening following Wilson et al. (2021). Females were mated in estrus at 3-4 months of age, and video recording was used to detect copulation during the night. Two individuals were used for sampling the implantation stage, 6.5 dpc and one was used as control 6 days after oestrus (early diestrus).

M.musculus (C57BL/6J) were maintained at the University of Vienna according to Institutional Animal Care protocols in a separate facility. The estrus cycle was monitored by vaginal swabbing following Ajayi & Akhigbe (2020). Copulation was determined by the presence of a copulatory plug and considered as day 0.5 post-copulation. Two individuals were used for sampling the implantation stage at 4.5 dpc and two were used for sampling the diestrus stage.

M. domestica were raised in a breeding colony at Yale University according to ethical protocols approved by the Yale University Institutional Animal Care and Use Committee (#2020-11313). Two individuals were used for sampling each stage: the non-cycling non-pregnant endometrium (n=2), 7.5 dpc (n=2) and 13.5 dpc (n=2, previously reported in Stadtmauer, Basanta et al., 2024). Video recording was used to assess the precise time of copulation. If multiple copulations were observed, the first was always used to establish 0 dpc.

Single-Cell Dissociation

Whole uterine horns were dissected into phosphate-buffered saline (PBS). Portions of ~ 0.2g per individual were minced into ~1 mm^3 cubes and transferred into a digestive solution containing 0.2 mg/mL Liberase TL (05401020001, Sigma) in 1800 µL PBS. The tissue was then incubated at 37ºC for 15 minutes and then passed 10 times through a 16-gauge needle attached to a 3-mL syringe. This process was repeated another two more times, the last time with a 18-gauge needle for complete dissociation. 2 mL of charcoal-stripped fetal bovine serum (100-199, Gemini) were added to stop digestion by inverting the tube several times. After that, the cell suspension was passed through a 70-µm cell strainer then a 40-µm cell strainer to get rid of any remaining chunks of tissue. The filtered cell suspension collected was centrifuged at 300 g for 5 minutes. The resulting pellet was resuspended in 1x ACK red blood cell lysis buffer (A1049201, Thermo-Fisher), incubated at room temperature for 5 minutes, and centrifuged again. The final pellet was resuspended in PBS containing 0.04% bovine serum albumen (A9647, Sigma) and Accumax (07921, Stem Cell Technologies). The resulting single cell suspension was assessed with a Cellometer (mouse, guinea pig) or hemacytometer (opossum) to assess cell concentration and viability with trypan blue stain. Only cell suspensions with viability higher than 80-85% (rodent) or 70% (opossum) were used.

Library preparation and sequencing

Cells were captured using the 10X Chromium platform (3' chemistry, version 3). All libraries were generated according to manufacturer protocols (CG000315). Mouse and guinea pig libraries were generated at the University of Vienna and opossum libraries were generated at the Yale Center for Genomic Analysis.

Libraries were sequenced using an Illumina NovaSeq by the Yale Center for Genomic Analysis (*M. domestica*) at a read depth exceeding 20,000 reads/cell and at the Next Generation Sequencing Facility of the Vienna Biocenter (*C. porcellus* and *M. musculus*) at a read depth of around 400M reads per sample (Illumina NovaSeq S4 PE150 XP for *C. porcellus* and Illumina NovaSeq SP Assymetric 10X for *M. musculus*).

Single-Cell Data Analysis

Sequencing reads were aligned to reference genomes using the 10X Genomics CellRanger software (≥v7.0.0). *Monodelphis domestica*, *Cavia porcellus*, and *Mus musculus*, were mapped to their

respective Ensembl genome annotations (ASM229v1 v104, cavPor3.0 v104, and GRCm39 v104, respectively).

Species-specific single-cell datasets were analyzed and annotated separately following seurat and scanpy standard functions and criteria to reveal species-specific cell types. Cells with fewer than 700 unique features or greater than 25% of transcripts of mitochondrial origin were filtered, as well as cells predicted to be doublets by doubletdetection (v4.2) (Gayoso & Shor, 2022). Library size normalization, log1p normalization, feature selection, dimensionality reduction, clustering and marker gene identification were performed using scanpy ≥ v1.9.1 (Wolf et al., 2018) for opossum and Seurat v4 (Hao et al., 2021) for mouse and guinea pig. Replicates belonging to the same species were corrected for batch effect using harmony (harmonypy, v0.0.9, r-harmony, v0.1) (Korsunsky et al., 2019), which adjusts principal components to aid in cluster delimitation but does not alter expression values. The optimal number of clusters was determined by comparison to those expressing unique gene expression confirmed by the use of cNMF v1.4.1 (Kotliar et al., 2019) gene expression module analysis. The optimal numbers of factors (K) were chosen based upon manual examination of stability-error curves as in Brückner et al. (2022).

Differential gene expression between pseudo-bulk transcriptomes of cell types from the same species at different time points was conducted using the DESeq2 method (v3.19, Love et al., 2014) and its python reimplementation pydeseq2 (v0.4.10, Muzellec et al., 2023). As luminal (LE) and glandular (GE) epithelial cluster cell abundances differed substantially across replicates, we merged all epithelial cell types into a single cluster ("eEpi") for the purposes of differential gene expression analysis. To identify differentially expressed ligands and receptors, the list of genes subjected to differential expression testing was subset to only genes encoding ligands, receptors, or the subunits of in our ligand-receptor ground truth database used for communication inference. Gene set enrichment on differentially expressed genes was conducted using gseapy (v1.1.3; Fang et al., 2023) against the gene sets of single-molecule perturbations on cultured cell lines available in the NCBI gene expression omnibus (Enrichr "Gene_Pertubations_from_GEO_up").

Time-matched pre-implantation blastocyst scRNA-seq data were obtained from public repositories. These included opossum E7.5 bilaminar blastocyst (EMBL ArrayExpress E-MTAB-7515) (Mahadevaiah et al., 2020), mouse E4.5 blastocysts (NCBI Gene Expression Omnibus GSE63266) (Nakamura et al., 2015), guinea pig E5.5 blastocysts (China National Center for Bioinformation PRJCA028188) (Guan et al., 2024), and human day 7 blastocysts (EMBL ArrayExpress E-MTAB-3929) (Petropoulos et al., 2016). Blastocyst cell types were annotated to identify trophectoderm using the original authors' annotations in the case of opossum and human. For mouse and guinea pig, where the authors did not provide original cell annotations, trophectoderm was identified as a *CDX2* and *GATA3*-expressing cell population following dimensional reduction and leiden clustering. Single-cell RNA-seq data from the human endometrium at mid-secretory stage (Marečková et al., 2024) and first trimester of pregnancy (Arutyunyan et al., 2023) were obtained from reproductivecellatlas.org.

Homology Inference by SAMap

Datasets from all species were integrated into a shared UMAP manifold using SAMap (v.1.3.4) (Tarashansky et al., 2021). Pairwise mapping scores between putative cell type clusters across species were calculated using the get_mapping_scores() function on the pooled transcriptome of all cells in each cluster. was used to calculate transcriptomic similarity scores between cells of different species.

Cell-Cell Communication Analysis

We inferred cell communication events between cell type transcriptomes using the method described in Stadtmauer, Basanta et al. (2024). Briefly, "human-equivalent transcriptomes" of each species were generated by mapping loci to their top BLAST hits using the BLAST+ graphs generated during the first stage of the SAMap pipeline. To maximize coverage, in cases of many:one human orthology, counts of all detected paralogs were pooled together. A ground truth ligand-receptor database was built as a manually extended fork of CellPhoneDB v5.0.0 (Garcia-Alonso et al., 2001) with additional curation and metadata. This modified list is archived at https://gitlab.com/dnjst/ViennaCPDB/. Cell interactions were inferred using expression thresholding with a cutoff of 0.2 (20% of cells in the cluster) for both ligand and receptor, or the least-expressed subunit if composed of multiple parts, using chinpy (v0.0.55; https://gitlab.com/dnjst/chinpy). Statistical testing for significantly cell type-enriched interactions were conducted using LIANA+ (v1.2.0) (Dimitrov et al., 2023).

For circos plots, depicted interactions are subset to those annotated as "Secreted Signaling" in our database, i.e. paracrine and endocrine peptide ligands, and "Small Molecule-Mediated", i.e. enzymes producing steroid hormones and other small molecules, but excluding extracellular matrix-mediated ligand-receptor interactions and those requiring direct cell-cell contact. For space reasons, the mouse and guinea pig interaction wheels were truncated to only the top 100 interactions as ordered by the LIANA+ "specificity_rank" metric. Differential expression analysis of ligands and receptors was subset only to Secreted Signaling peptides.

For Figure 9c, Pearson correlations were calculated between all mid-gestation cell type secreted signal repertoires (Stadtmauer, Basanta et al., 2024) and the peri-implantation uterine epithelium signaling repertoires (this study) using the corr() function of the pandas (v2.2.2) package. As with differential gene expression, uterine epithelial cell secretomes were generated from pooled populations of glandular and luminal sub-types ("eEpi"). Input data were boolean matrices of all ligands classified as Secreted Signaling (a total of 302), with a threshold proportion of cells in the cluster to be considered "on" of 0.10. Venn diagrams were plotted using the matplotlib_venn (v1.1.1) package and Jaccard indices of sets were calculated via the formula $J(A,B) = |A \cap B| / |A \cup B|$.

Differential Expression Analysis

To identify differentially expressed ligand and receptor signaling from epithelial to stromal cells at implantation, differential gene expression analysis was performed using pyDESeq2 (v0.4.10; Muzellec et al., 2023) on pseudo-bulk transcriptomes of peri-implantation and non-pregnant control stages grouped by cell type and stage (decoupleR-py v1.8.0; Badia-i-Mompel et al., 2022). Significantly changed genes were classified as those with a Benjamini-Hochberg adjusted Wald test p-value of less than 0.05. Only ligands with log2 fold-change values of mean ligand expression greater than 0.1 were included in Table S1.

Materials and methods citations

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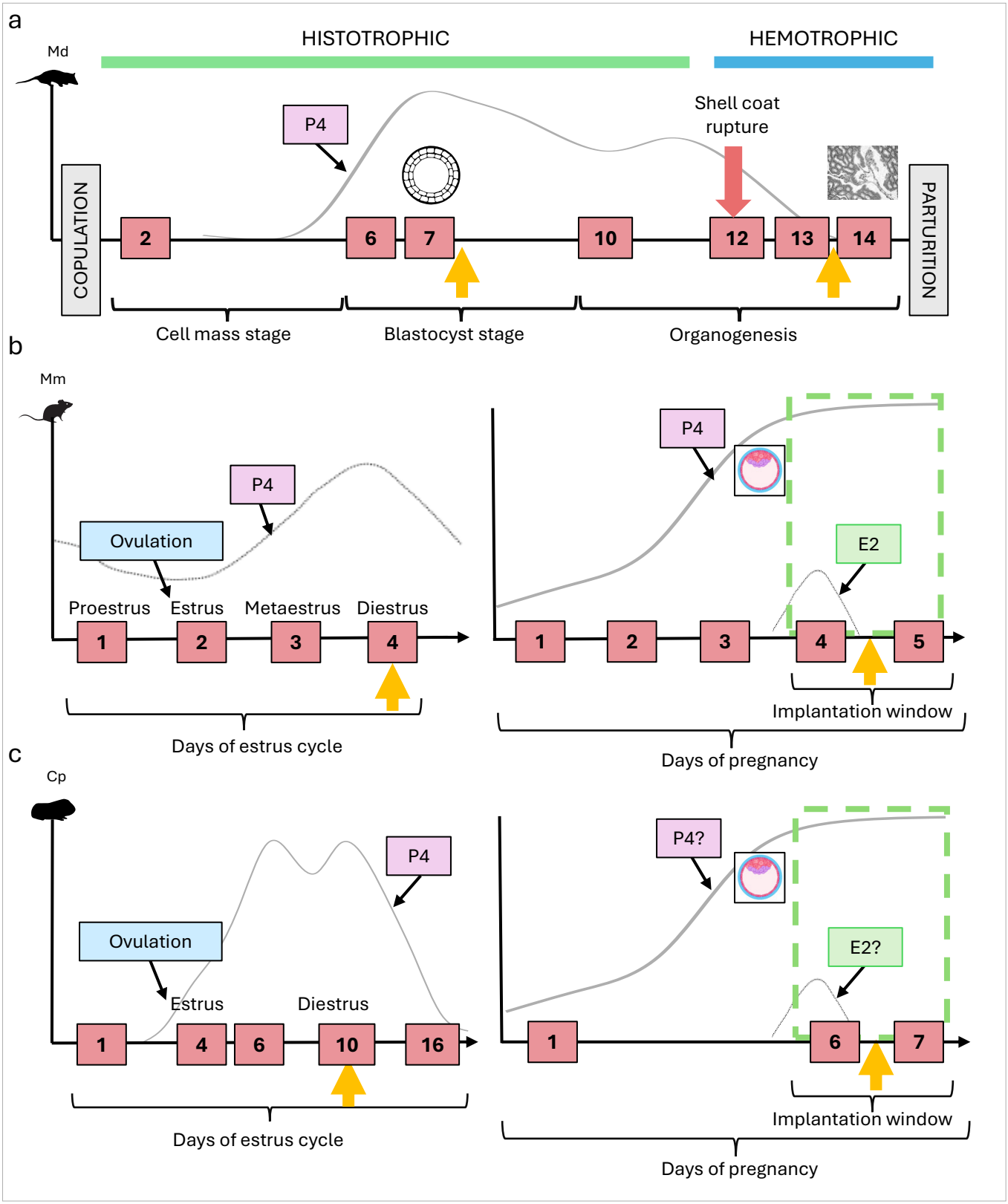

Figure 1. a) Diagram showing the gestation timeline of the opossum (adapted from Hansen (2017)). Yellow arrows indicate the days 7.5dpc and 13.5dpc included in our study. Progesterone peak at day 7 as reported by Hinds et al. (1992) **b)** Diagram showing mouse estrus cycle and window of implantation. Yellow arrows indicate the diestrus and implantation day (4.5dpc) used in the study. Progesterone patterns during first days of pregnancy as reported in Virgo & Bellward (1974) **c)** Diagram showing guinea pig estrus cycle and window of implantation. Yellow arrows indicate the diestrus (day 6 after ovulation) and implantation day (6.5 dpc) used in the study. Progesterone peak during the oestrus cycle as reported by Challis et al. (1971). Days of the estrus cycle in the guinea pig are approximate (proestrus lasts 2-4 days, the estrus 11h, metaestrus 4 days and diestrus from 8 to 19 days). P4 = progesterone, E2 = estrogen. Blastocyst icons and rest of hormonal patterns from BioRender. Mm= *Mus musculus*, Cp = *Cavia porcellus*, Md= *Monodelphis domestica*.

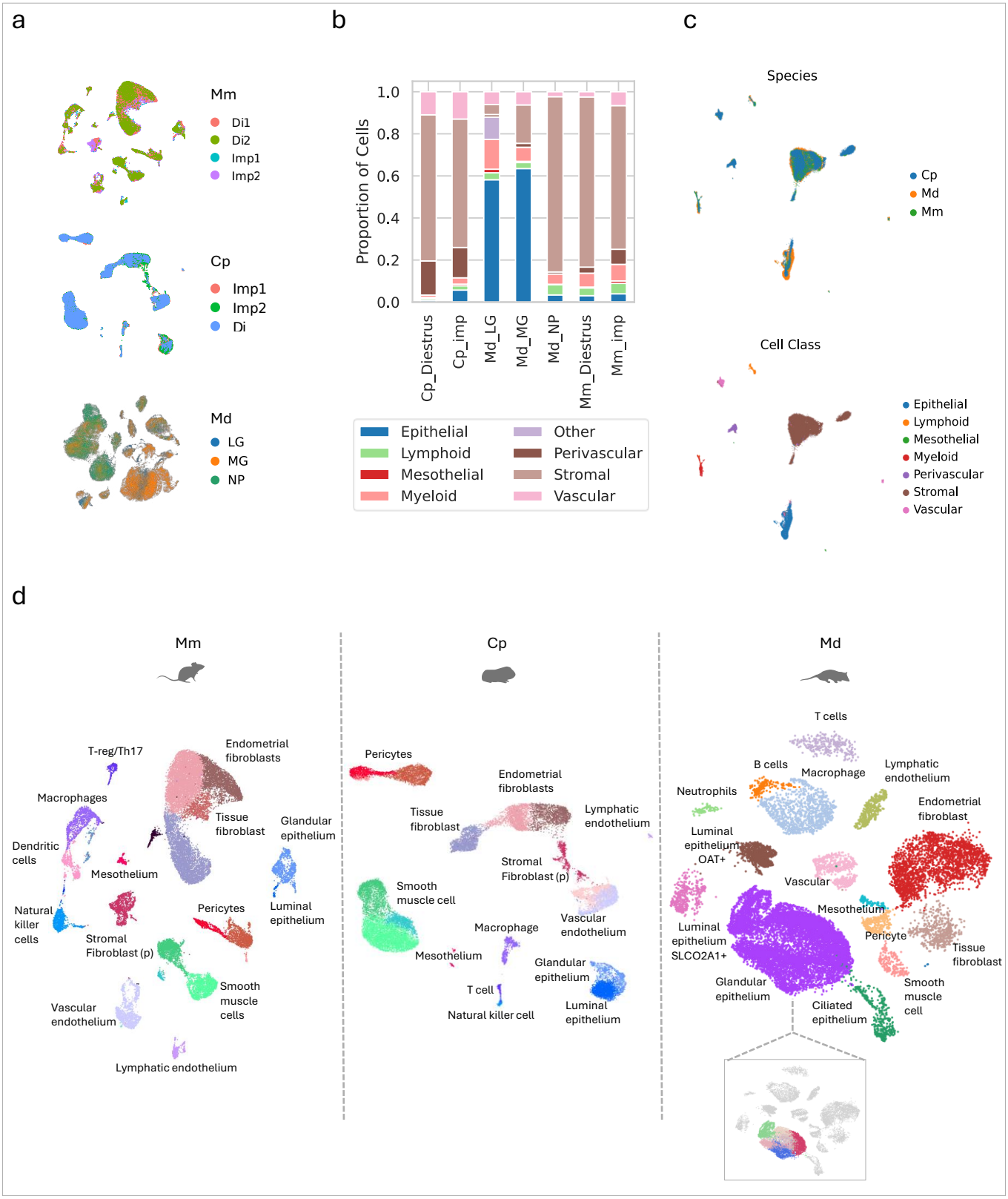

Figure 2. a) Harmony integration of samples (Imp= implantation, Di= diestrus, LG= late-gestation, MG= mid-gestation, NP= non-pregnant) within each species (Mm= *Mus musculus,* Cp= *Cavia porcellus*, Md= *Monodelphis domestica*) **b)** Relative cell class abundance per stage and in each species **c)** Major cell classes shared in the three species according to SAMap **d)** Individual UMAPs showing cell cluster identities resulting from the integration of pregnant and non-pregnant samples in mouse, guinea pig and opossum, along with the multiplicity of glandular clusters in the latter (for simplicity, placental cells present at late-gestation opossum are not shown). (p) = proliferating.

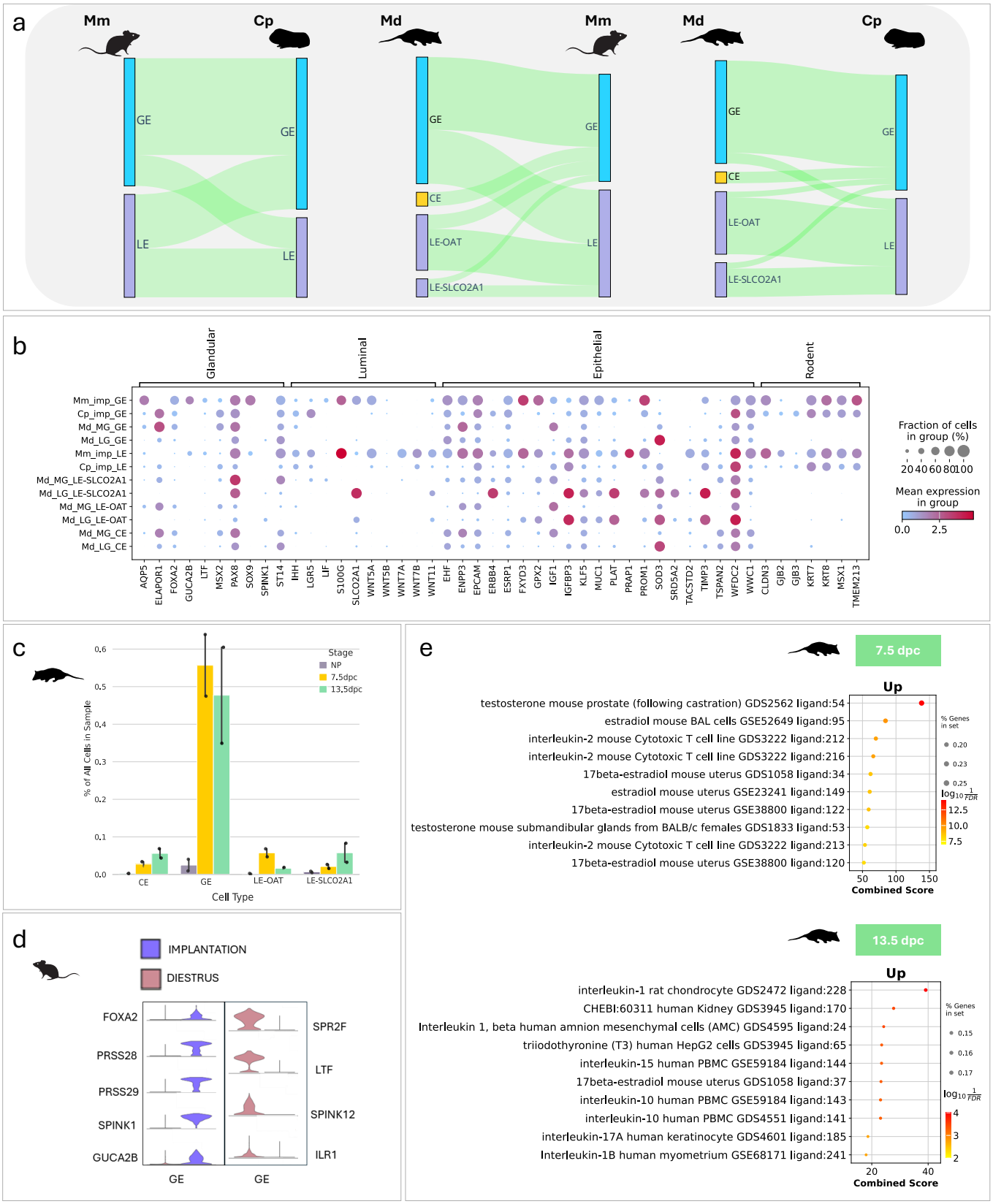

Figure 3. Epithelial cell type diversity, homology and gene expression dynamics. a) Sankey plots with bands proportial to SAMap transcriptomic similarity scores (0-1) of epithelial celll types across species **b)** Selected mouse, guinea pig and oposum epithelial maker genes at implantation (imp), oposum mid-gestation (MG) and late-gestation (LG). GE= glandular epithelium, LE= luminal epithelium, CE= ciliated epithelium. **c)** Abundance of the different epitelial cell types in the oposum across stages **d)** Differential expressed genes in mouse epithelial gland expression between implantation and diestrus **e)** Gene enrichment analysis on differentially expressed genes in the opossum glands between non-pregnant and 7.5 dpc and non-pregnant and 13.5 dpc using the database from EnrichR "Gene Perturbations from GEO up" gene set.

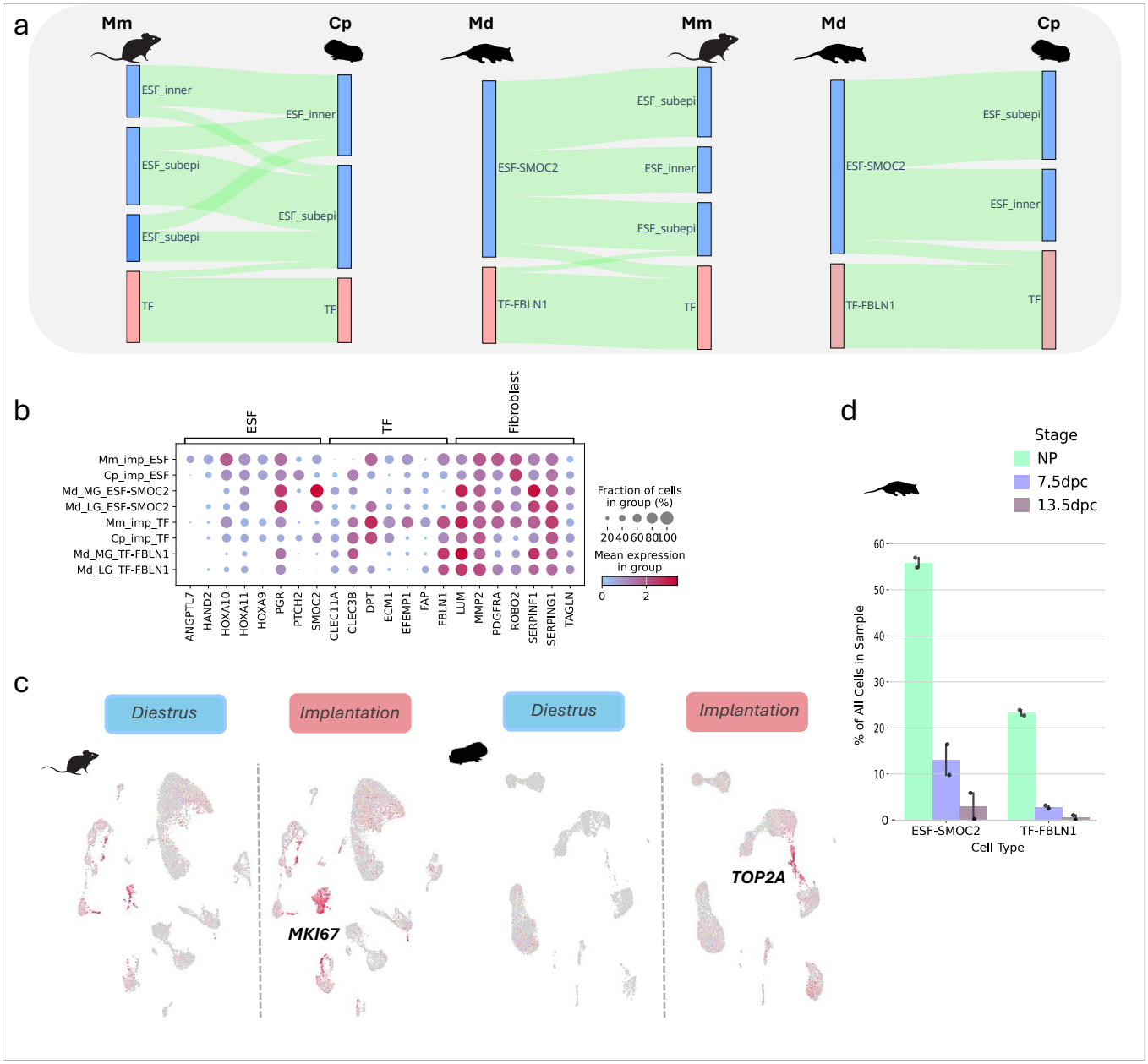

Figure 4. Fibroblast diversity, homology and abundance dynamics. a) Sankey plots showing SAMap transcriptomic similarity of the stromal cells across species. Thickness of the bands correspond to SAMap similarity scores (0-1) **b)** Expression of mice, guinea pig ad opossum fibroblast marker genes at same stages as in Figure 3b. ESF= endometrial stromal fibroblast, TF = tissue fibroblast **c)** Feature plots in rodents showing the proliferation of ESFs in implantation compared to diestrus through the expression of *MKI67* and *TOP2A* **d)** Barplot showing the abrupt decrease in stromal abundance towards late-gestation in the opossum. NP= non-pregnant.

	Mm 4.5 dpc	Cp 6.5 dpc	Md 7.5 dpc	Md 13.5 dpc
IHH- PTCH1	✓↑	✓↑	✓↑	X
LIF-LIFR	✓↑	X	✓↓	X
WNT-FZD	✓	✓	✓↓	X

Figure 5. Summary of upregulated and downregulated interactions from the epithelium to the endometrial stromal fibroblast compared to diestrus stages (in rodents) and the opossum non-pregnant/non-cycling endometrium. Detailed information in Table S4.

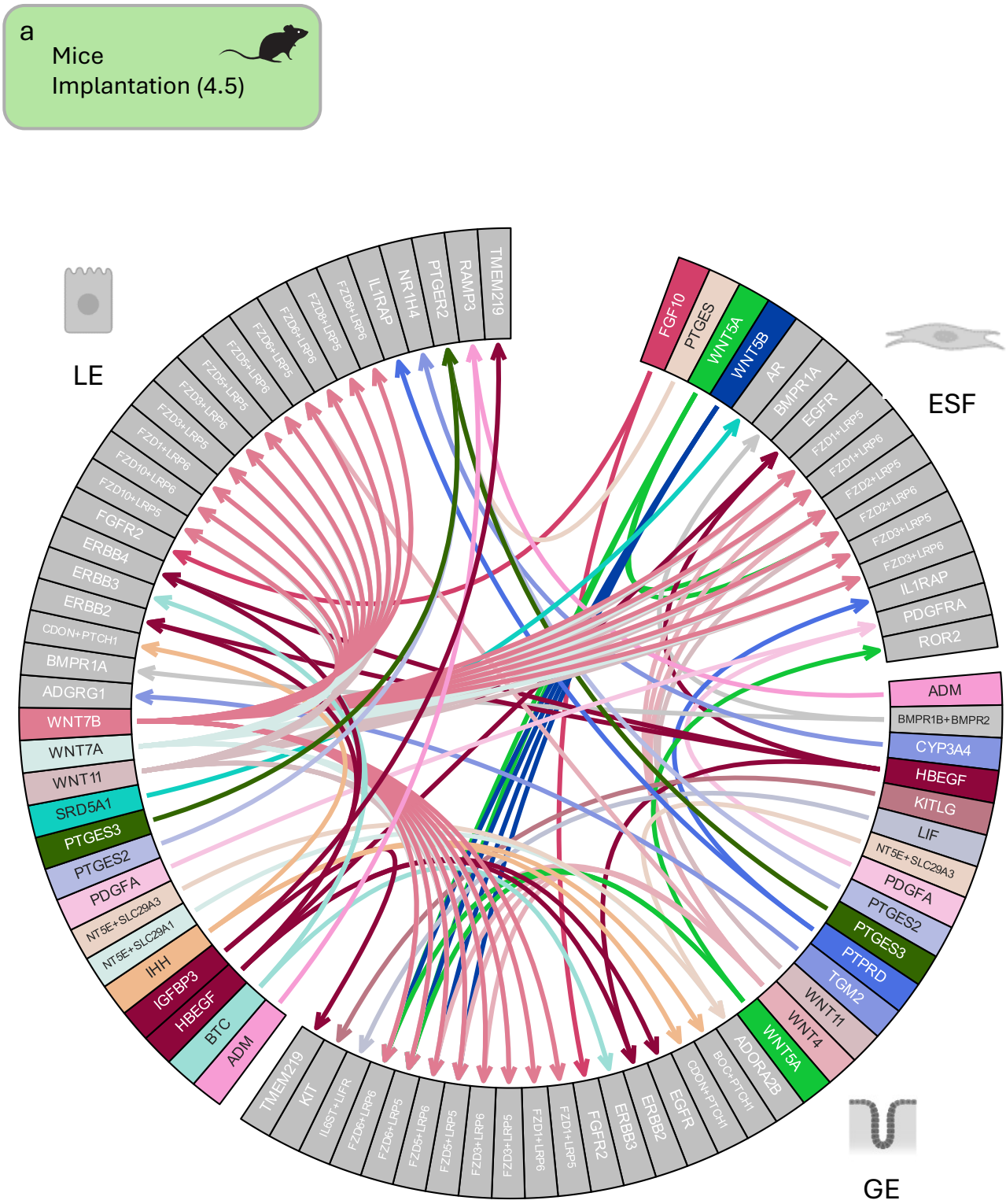

Figure 6. Mouse epithelial-stromal crosstalk. a) Circos lianaplots showing the first 125 putative (secreted-only) interactions sorted according to specificity rank involving the epithelial- stromal crosstalk at mouse peri-implantation. Ligands are shown in color and receptors in grey. Cell type icons from BioRender. LE = luminal epithelium. ESF = endometrial stromal fibroblast, GE = glandular epithelium.

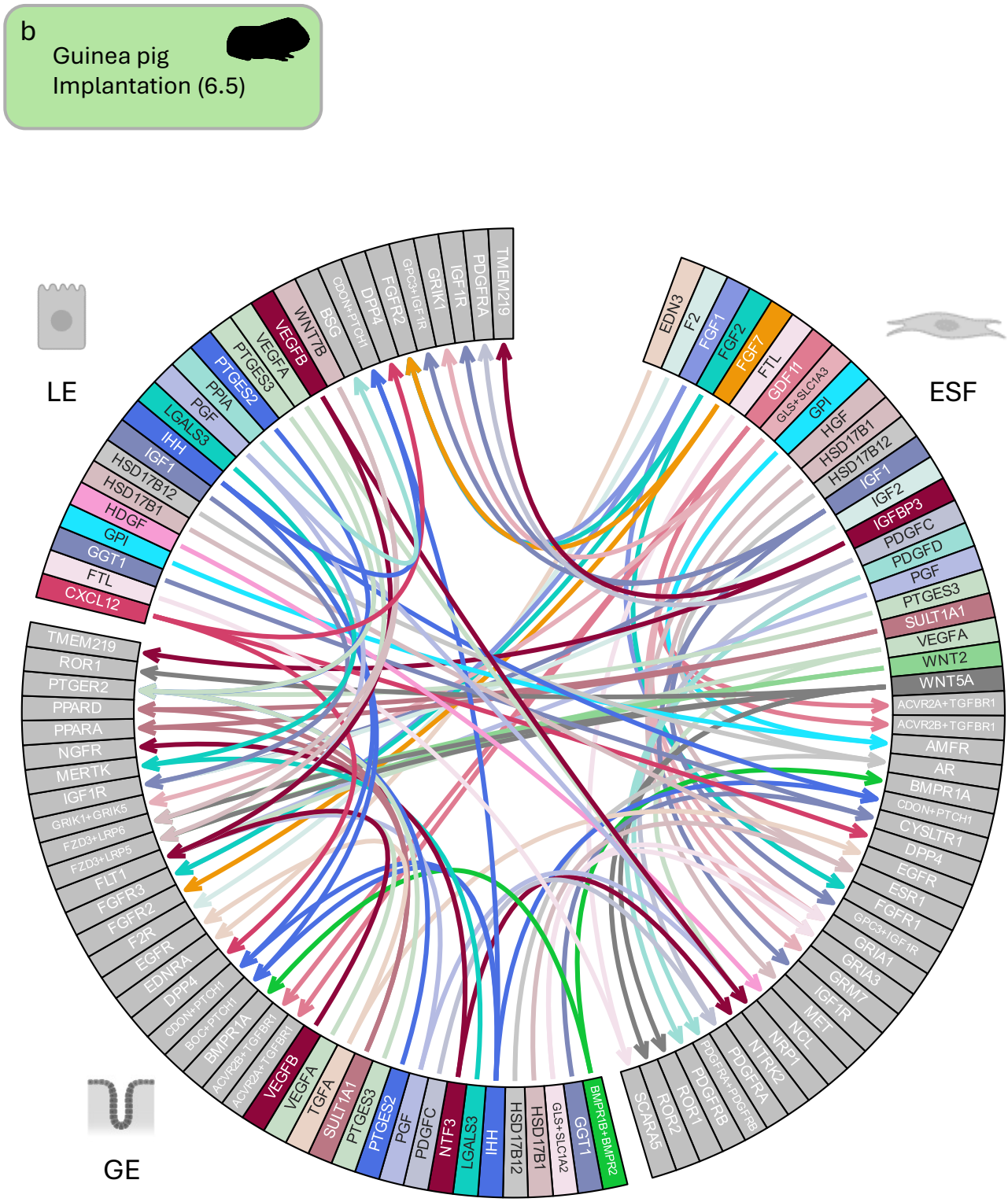

Figure 6. Guinea pig epithelial-stromal crosstalk. b) Circos lianaplots showing the first 125 putative (secreted-only) interactions sorted according to specificity rank involving the epithelial- stromal crosstalk at guinea pig peri-implantation. Ligands are shown in color and receptors in grey. Cell type icons from BioRender. LE = luminal epithelium. ESF = endometrial stromal fibroblast, GE = glandular epithelium.

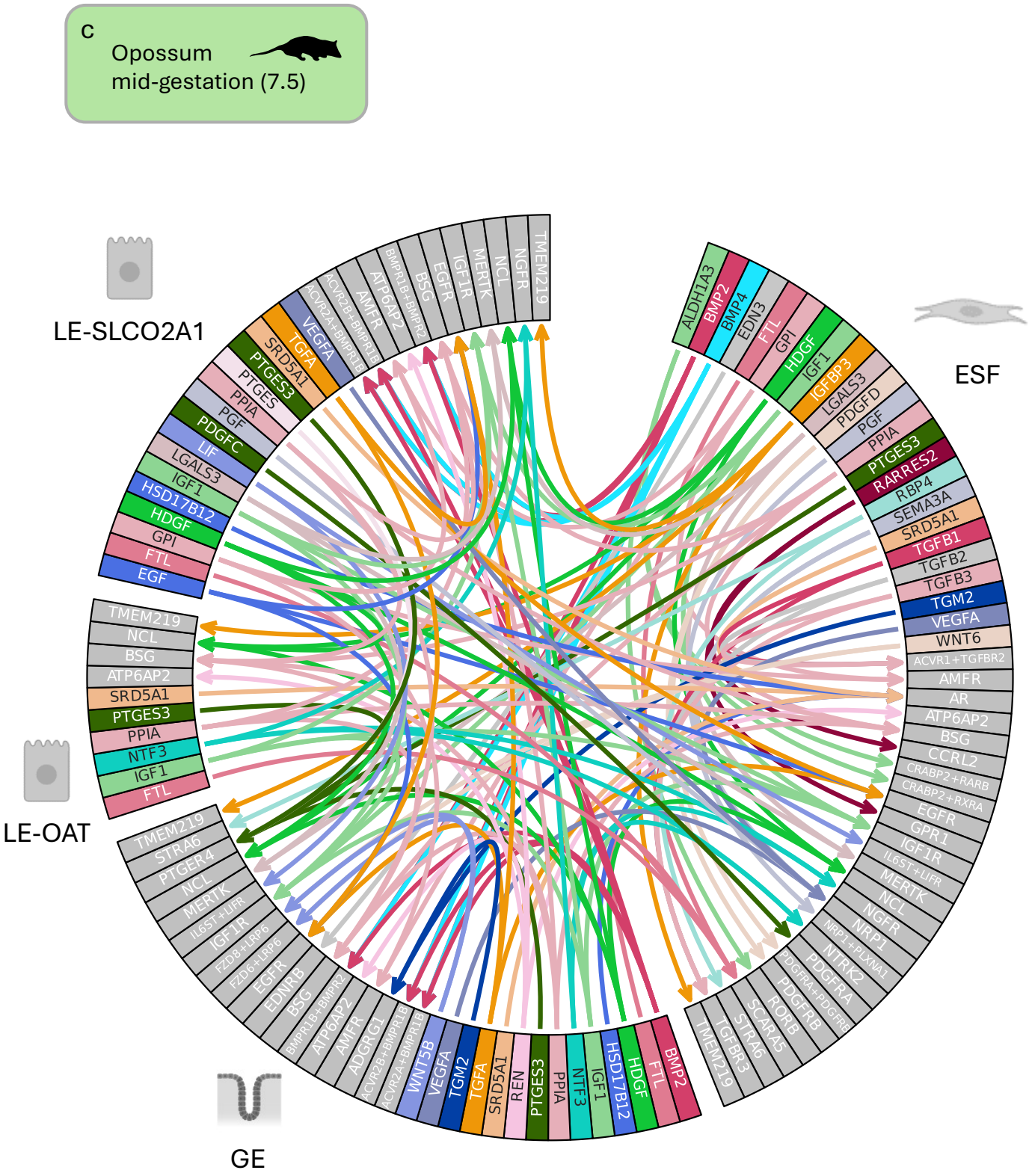

Figure 6. Mid-gestation opossum epithelial-stromal crosstalk. c) All the interactions (secreted-only) above threshold present at mid-gestation opossum (81). Ligands are shown in color and receptors in grey. Cell type icons from BioRender. Ligands are shown in color and receptors in grey. Cell type icons from BioRender. LE = luminal epithelium. ESF = endometrial stromal fibroblast, GE = glandular epithelium.

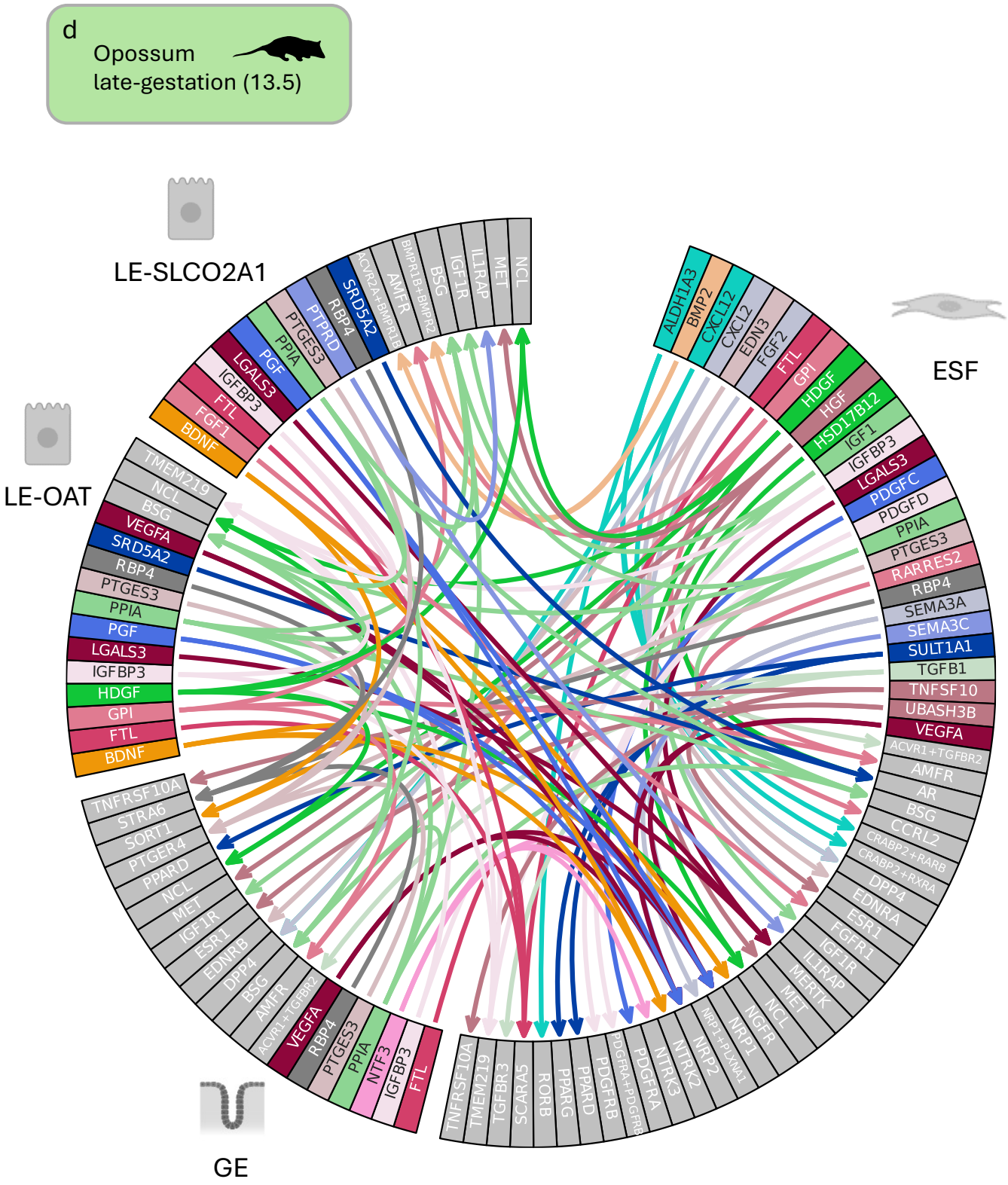

Figure 6. Late-gestation opossum epithelial-stromal crosstalk. d) All the interactions (secreted-only) above threshold present at late-gestation opossum (121). Ligands are shown in color and receptors in grey. Cell type icons from BioRender. Ligands are shown in color and receptors in grey. Cell type icons from BioRender. LE = luminal epithelium. ESF = endometrial stromal fibroblast, GE = glandular epithelium.

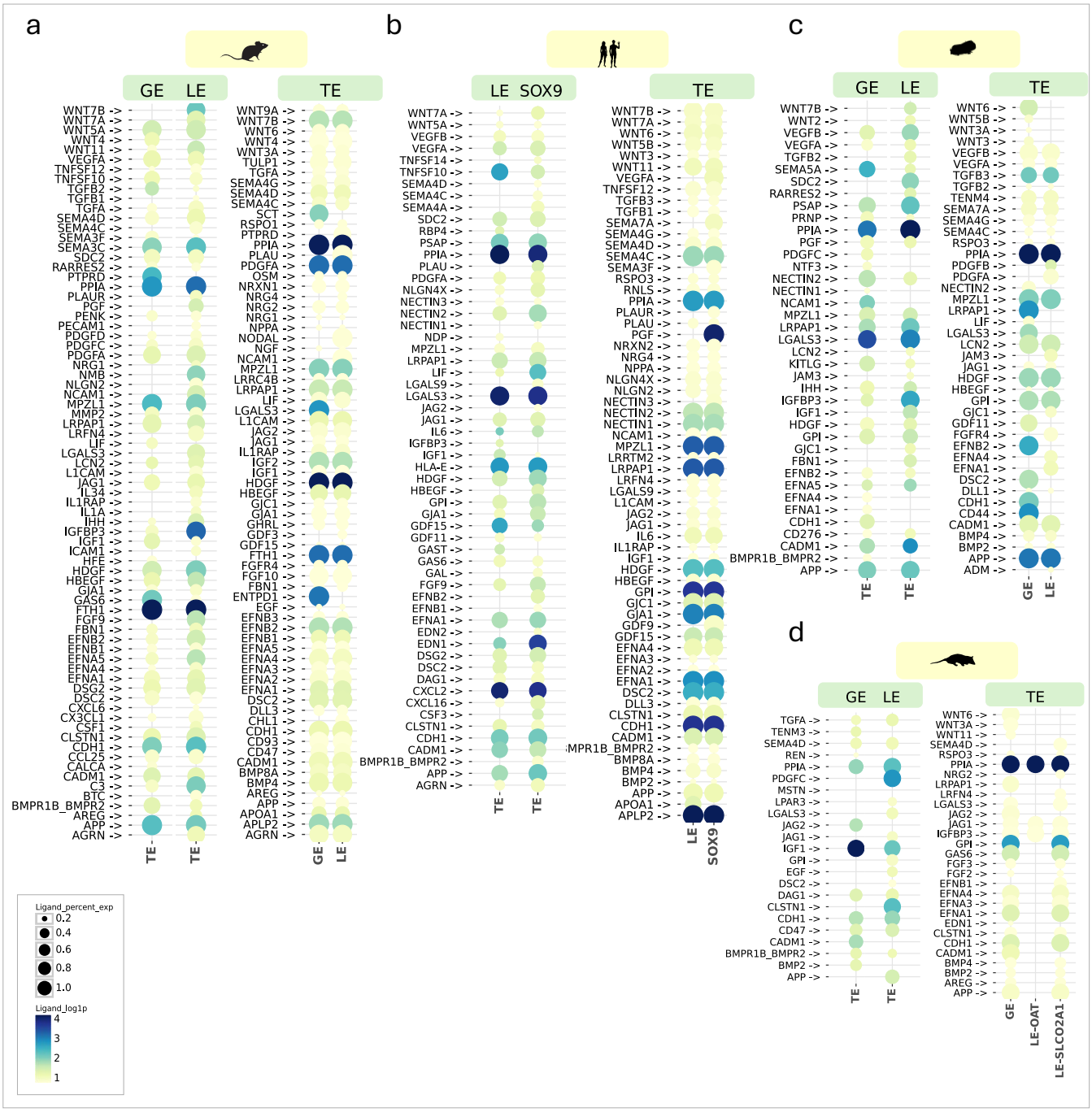

Figure 7. Dotplots showing inferred ligands from epithelial cells to the trophoectoderm (left) and from the trophoectoderm to epitelial cells (right) between in **a)** mouse **b)** human **c)** guinea pig and **d)** opossum. Interactions include cell-cell contact and ECM. All interactions showed passed the 20% expression threshold. TE = trophoectoderm, SOX9= human epithelial subtype as identified in (Marečková et al., 2024), LE= luminal epithelium, GE = glandular epithelium.

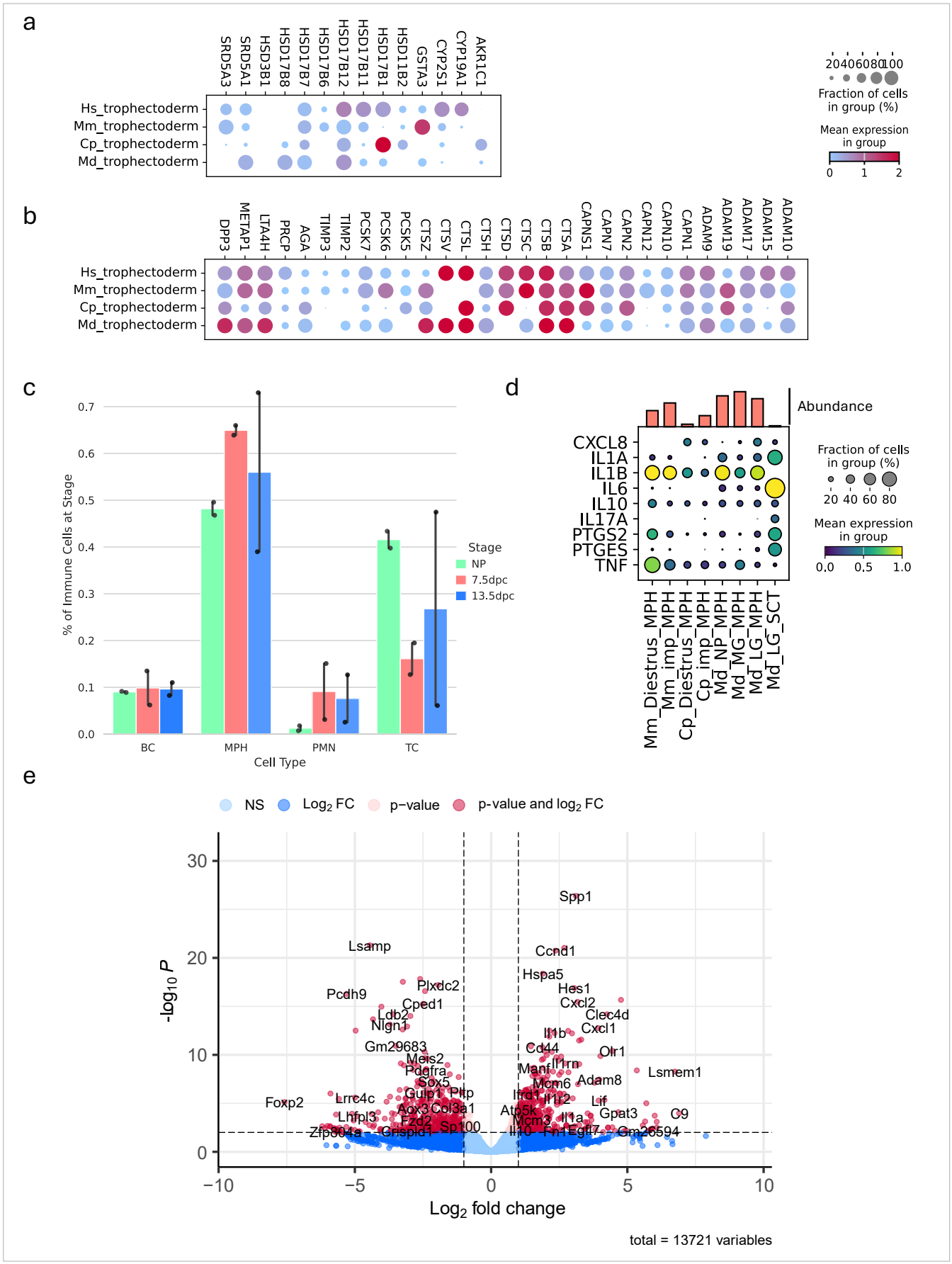

Figure 8. Blastocyst expression of **a)** small mediators involved in steroidogenesis and **b)** proteases in the human, mouse, guinea pig and oposum trophoectoderm **c)** Abundance of different immune cell types across stages in the opossum **d)** Expression of inflammatory mediators in macrophages (MPH) in different stages across species and in the opossum syncytium (SCT). Bars on top represent cell abundance **e)** Volcano plot showing differential expression in mouse macrophages in implantation compared to diestrus.

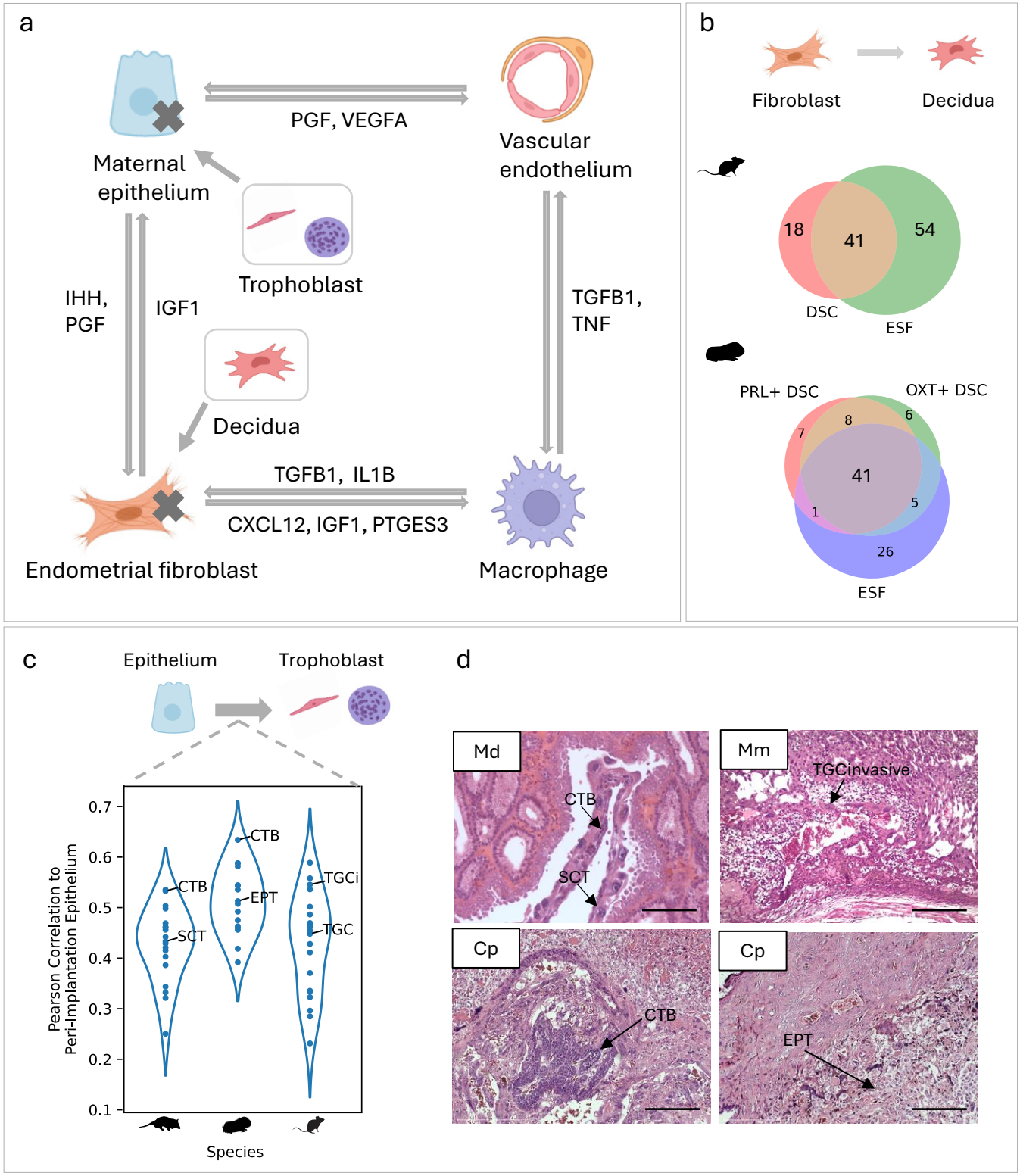

Figure 9. Signaling continuity between implantation and placentation. a) Conserved signaling interactions between the luminal epithelium, endothelium, macrophage and fibroblast in rodents and opossum, also showing the replacement of epithelial and fibroblast maternal cell types by the trophoblast and decidua during the transition to invasive placentation **b)** Venn diagram showing ligand expression overlap between ESF to decidua from implantation to mid-gestation in rodents **c)** Pearson correlation between the ligands expressed by the maternal epithelium and trophoblast populations of the same species, including the opossum **d)** Hematoxylin & Eosin showing the location of the same trophoblast populations across species. All scale bars correspond to 200 µm. DSD = decidual cell CTB = cytotrophoblast, SCT= syncytiotrophoblast, EPT = extraplacental trophoblast, TGCi = invasive giant trophoblast, TGC= giant trophoblast. Cell type icons from BioRender.

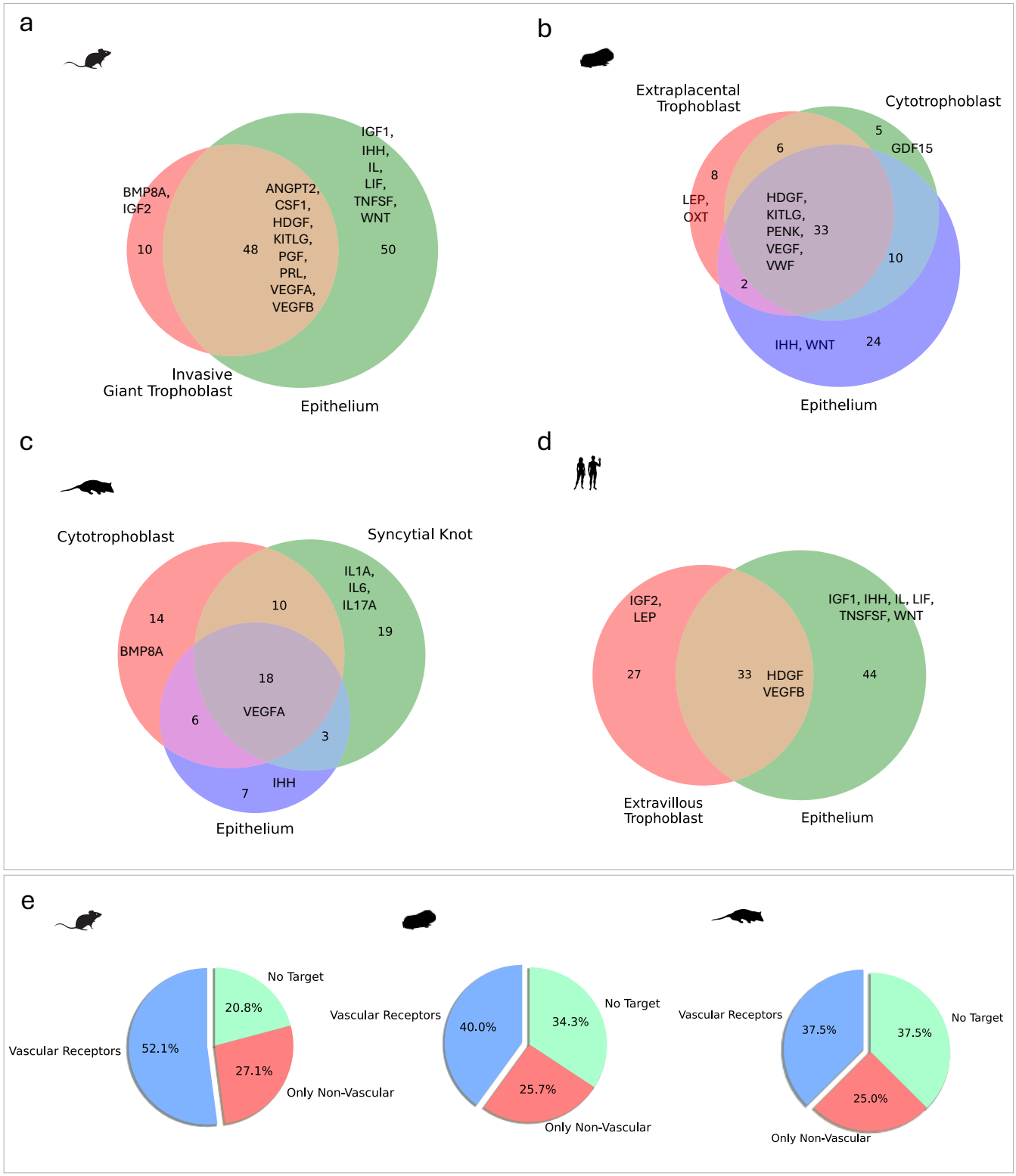

Figure 10. a) Venn diagram of the maternal epithelium of mouse peri-implantation and invasive trophoblast **b)** Venn diagram of the maternal epithelium of guinea pig peri-implantation and invasive trophoblast **c)** Venn diagram of the opossum day 7.5 epithelium and placental types at 13.5 dpc **d)** Venn diagram of the maternal epithelium and the human extravillous trophoblast **e)** Pie Charts showing the percentage of ligands with vascular receptors in mouse, guinea pig and opossum.

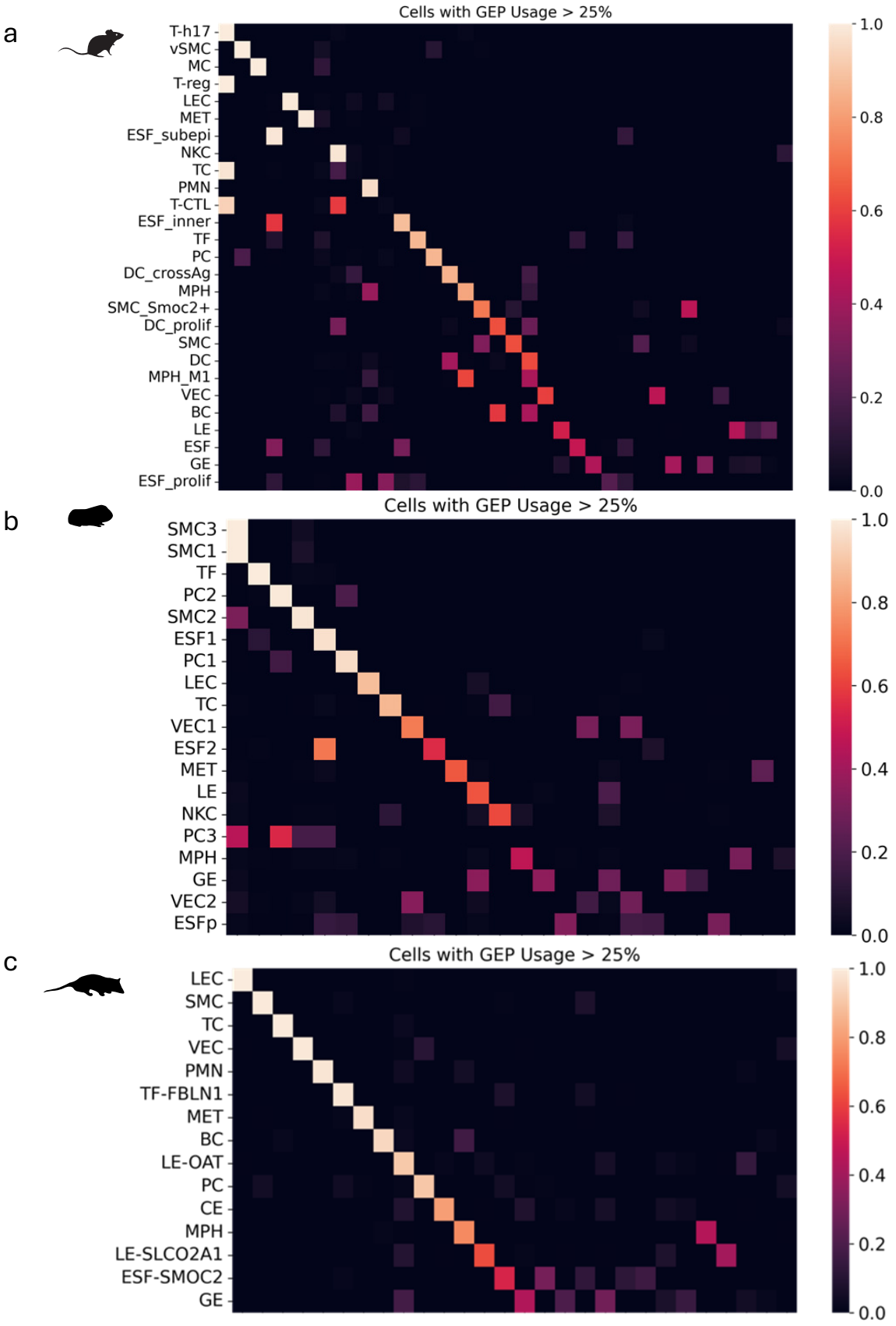

Figure S1. cNMF heatmaps showing percentage usage of gene expression programs (rows) in each maternal cell type in **(a)** mouse **(b)** guinea pig **(c)** and the opossum after harmony integration of stages within species.

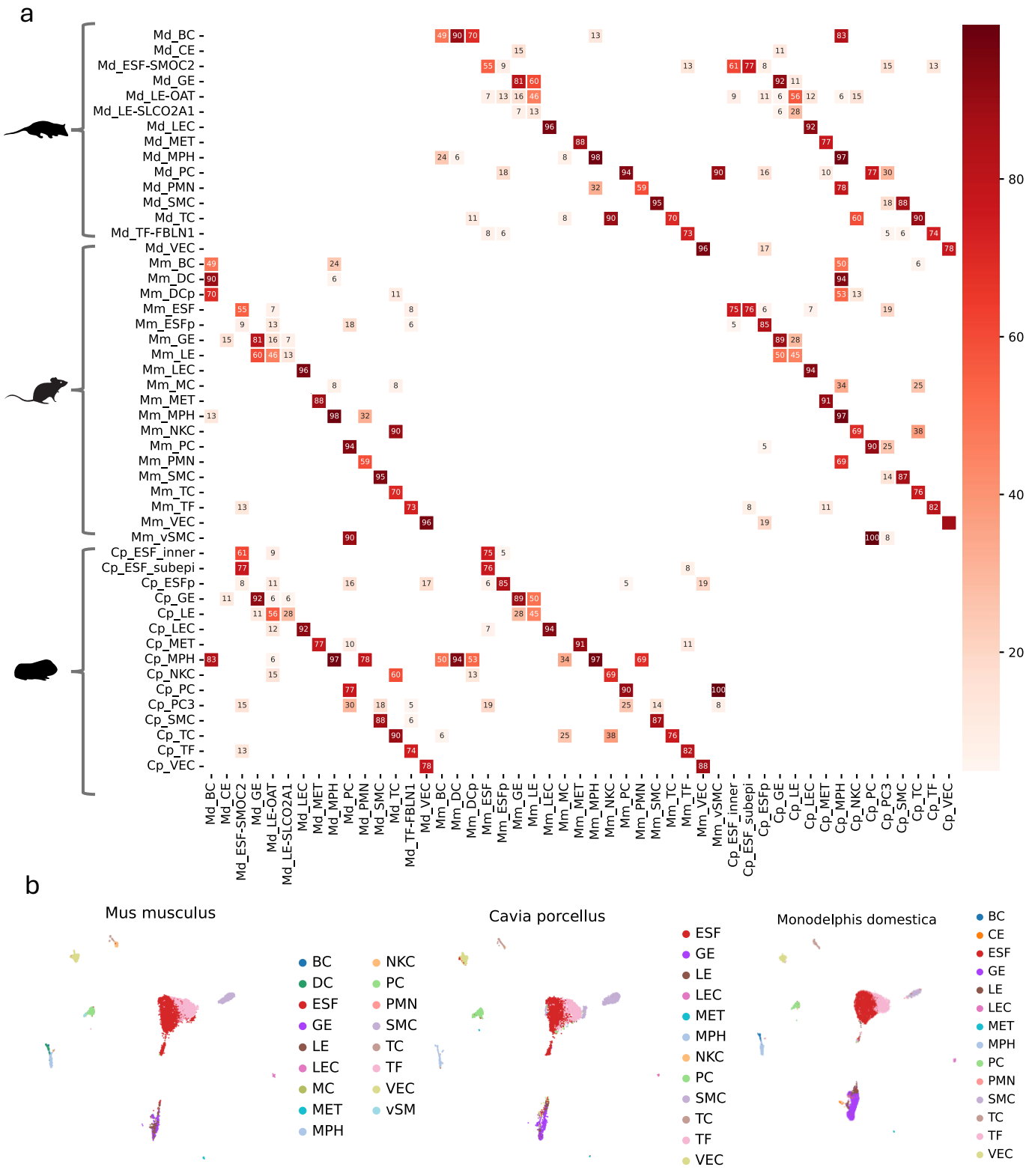

Figure S2. **a)** SAMap heatmap showing transcriptomic similarity scores across species **b)** Shared UMAP manifold across species, colored by the cell type as identified in the original, species-specific annotation, and showing correspondence across species. BC= B cell, DC= dendritic cell, ESF= endometrial stromal fibroblast, GE= glandular epithelium, LE = luminal epithelium, LEC = lymphatic endothelium, MC= mast cell, MET= mesothelial cell, MPH= macrophage, NKC= natural killer cell, PC= pericyte, PMN = polymorphonueclar neutrophils, SMC= smooth muscle cell, TC= T cell, TF = tissue fibroblast, VEC= vascular endothelium, vSM= vascular smooth muscle, CE= ciliated cell.

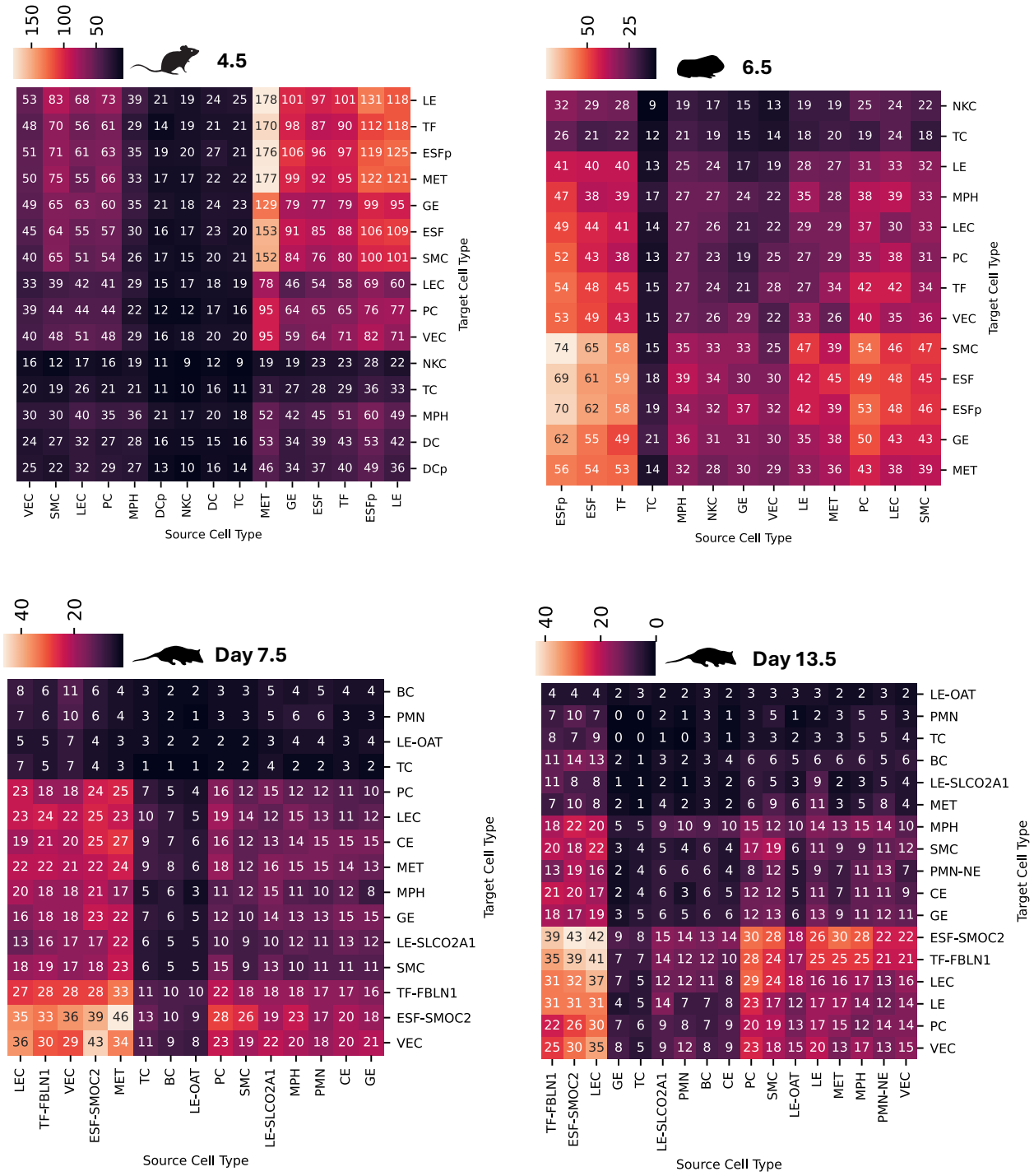

Figure S3. Heatmaps showing the numer of predicted secreted interactions above the 20% threshold between all clusters in mouse and guinea pig implantation (day 4.5 and 6.5 respectively), as well as in mid (day 7.5) and late-gestation opossum (day 13.5).

ligand	receptor	ligand_log2FC	receptor_log2FC	ligand_padj	receptor_padj
WNT7A*	FZD9_LRP5	-3.06	-0.07	2.34E-08	9.58E-01
	SFRP5*	-3.06	5.71	2.34E-08	2.51E-05
	FZD2_LRP6	-3.06	-0.29	2.34E-08	7.76E-01
	FZD2_LRP5	-3.06	-0.29	2.34E-08	7.76E-01
	FZD8_LRP6	-3.06	-0.32	2.34E-08	7.36E-01
	FZD3_LRP6	-3.06	0.22	2.34E-08	8.35E-01
	FZD8_LRP5	-3.06	-0.32	2.34E-08	7.36E-01
	FZD3_LRP5	-3.06	0.22	2.34E-08	8.35E-01
	FZD4_LRP5	-3.06	0.32	2.34E-08	7.50E-01
	FZD9_LRP6	-3.06	-0.07	2.34E-08	9.58E-01
	FZD1_LRP5*	-3.06	2.46	2.34E-08	8.52E-07
	FZD7_LRP6	-3.06	0.48	2.34E-08	5.46E-01
	FZD6_LRP5	-3.06	0.33	2.34E-08	7.27E-01
	FZD1_LRP6*	-3.06	2.46	2.34E-08	8.52E-07
	FZD4_LRP6	-3.06	0.32	2.34E-08	7.50E-01
	SFRP2	-3.06	0.19	2.34E-08	8.31E-01
	FZD7_LRP5	-3.06	0.48	2.34E-08	5.46E-01
	SFRP4*	-3.06	3.35	2.34E-08	1.64E-09
	FZD6_LRP6	-3.06	0.33	2.34E-08	7.27E-01
	SFRP1*	-3.06	5.64	2.34E-08	1.31E-11
	FZD10_LRP6	-3.06	0.61	2.34E-08	3.02E-01
	FZD10_LRP5	-3.06	0.61	2.34E-08	3.02E-01
	FZD5_LRP6	-3.06	-0.36	2.34E-08	6.95E-01
	FRZB*	-3.06	1.50	2.34E-08	5.79E-03
	FZD5_LRP5	-3.06	-0.36	2.34E-08	6.95E-01
GDF15*	ERBB2	-2.51	0.42	5.40E-05	5.66E-01
	CD48	-2.51	0.92	5.40E-05	2.11E-01
BMP5*	ACVR2B_BMPR1B	-2.31	0.32	2.40E-02	7.10E-01
	BMPR1B_BMPR2*	-2.31	2.83	2.40E-02	1.97E-05
	ACVR2A_BMPR1A	-2.31	0.03	2.40E-02	9.96E-01
	ACVR2A_BMPR1B	-2.31	0.03	2.40E-02	9.96E-01
	BMPR1A_BMPR2	-2.31	-0.41	2.40E-02	5.57E-01
	ACVR1_ACVR2B	-2.31	-0.28	2.40E-02	7.89E-01
	ACVR1_BMPR2	-2.31	-0.28	2.40E-02	7.89E-01
	ACVR1_ACVR2A	-2.31	-0.28	2.40E-02	7.89E-01
	ACVR2B_BMPR1A	-2.31	0.32	2.40E-02	7.10E-01
IL36B*	IL1RAP_IL1RL2	-2.29	-0.20	1.21E-02	8.60E-01
C3*	ITGAM_ITGB2	-2.23	0.50	4.12E-03	5.27E-01
	C3AR1	-2.23	0.35	4.12E-03	6.76E-01
	C5AR2	-2.23	0.17	4.12E-03	
PTN*	PTPRZ1	-2.15	-0.65	7.29E-04	3.46E-01
	ALK*	-2.15	2.29	7.29E-04	1.80E-03
TNFSF13*	TNFRSF17	-2.13	0.17	1.55E-03	
	TNFRSF13B	-2.13	0.06	1.55E-03	9.68E-01
LGALS3*	MERTK	-2.13	-0.28	5.85E-06	8.41E-01
PTHLH*	PTH2R	-2.05	-0.04	2.08E-02	9.85E-01

ligand	receptor	ligand_log2FC	receptor_log2FC	ligand_padj	receptor_padj
	PTH1R*	-2.05	-1.32	2.08E-02	8.71E-03
FGF1*	FGFR2*	-1.92	-1.32	2.67E-02	1.66E-02
	FGFR4*	-1.92	-1.22	2.67E-02	3.89E-02
	FGFR3	-1.92	-0.05	2.67E-02	9.77E-01
	FGFR1	-1.92	0.37	2.67E-02	6.02E-01
	ITGAV_ITGB3	-1.92	-0.47	2.67E-02	4.89E-01
PLAU*	PLAUR	-1.87	0.11	4.48E-02	9.45E-01
PDGFB*	PDGFRB	-1.85	-0.91	3.45E-02	8.36E-02
	PDGFRA	-1.85	0.86	3.45E-02	2.56E-01
	PDGFRA_PDGFRB	-1.85	0.86	3.45E-02	2.56E-01
AGRN*	LRP4_MUSK	-1.68	0.69	8.33E-03	2.92E-01
	PTPRS	-1.68	-0.90	8.33E-03	6.46E-02
JAG2	VASN	-1.29	-0.20	1.00E-01	8.51E-01
ANXA1*	FPR2	-1.24	-0.19	1.58E-02	8.55E-01
CXCL3	CXCR2	-1.23	0.05	1.16E-01	9.78E-01
PPIA*	BSG	-1.18	0.10	6.19E-03	9.20E-01
PDGFD	PDGFRB	-1.11	-0.91	5.30E-02	8.36E-02
	PDGFRA_PDGFRB	-1.11	0.86	5.30E-02	2.56E-01
CX3CL1	CX3CR1	-1.10	0.35	6.51E-02	6.81E-01
SEMA3F	NRP2	-1.05	0.27	5.08E-02	7.71E-01
CSF3	CSF3R	-1.02	0.21	1.73E-01	7.81E-01
ASIP	MC1R	-0.99	-0.33	1.66E-01	7.26E-01
CXCL16	CXCR6	-0.89	0.05	1.78E-01	9.78E-01
HDGF	NCL	-0.84	0.48	7.74E-02	7.25E-01
BMP8A	ACVR2B_BMPR1A	-0.83	0.32	2.33E-01	7.10E-01
	ACVR2A_BMPR1A	-0.83	0.03	2.33E-01	9.96E-01
	ACVR2A_BMPR1B	-0.83	0.03	2.33E-01	9.96E-01
	BMPR1A_BMPR2	-0.83	-0.41	2.33E-01	5.57E-01
	ACVR1_ACVR2A	-0.83	-0.28	2.33E-01	7.89E-01
	ACVR2B_BMPR1B	-0.83	0.32	2.33E-01	7.10E-01
	ACVR1_ACVR2B	-0.83	-0.28	2.33E-01	7.89E-01
	ACVR1_BMPR2	-0.83	-0.28	2.33E-01	7.89E-01
	BMPR1B_BMPR2*	-0.83	2.83	2.33E-01	1.97E-05
TNFSF9	TNFRSF9	-0.79	0.86	2.44E-01	2.21E-01
RNASET2	TLR8	-0.73	0.08	1.97E-01	
TAX1BP3	ADGRB2	-0.69	0.55	1.83E-01	4.42E-01
	ADGRB1*	-0.69	-2.54	1.83E-01	7.09E-03
LCN2*	SLC22A17	-0.68	0.01	2.03E-02	9.95E-01
NRTN	GFRA2_RET	-0.62	0.25	3.44E-01	8.25E-01
IL33	IL1RAP_IL1RL1	-0.61	-0.20	3.71E-01	8.60E-01
EDN1	EDNRA*	-0.57	1.82	3.53E-01	3.49E-04
	EDNRB*	-0.57	3.93	3.53E-01	1.13E-10
EDN2	EDNRA*	-0.53	1.82	3.93E-01	3.49E-04
	EDNRB*	-0.53	3.93	3.93E-01	1.13E-10
GPI	AMFR	-0.53	-0.39	2.88E-01	5.55E-01

ligand	receptor	ligand_log2FC	receptor_log2FC	ligand_padj	receptor_padj
CCL11	DPP4*	-0.53	3.55	3.80E-01	1.16E-04
	ACKR2*	-0.53	-2.98	3.80E-01	4.62E-03
SAA1	FPR2	-0.52	-0.19	3.37E-01	8.55E-01
KITLG	KIT	-0.51	-1.21	4.16E-01	1.05E-01
TGFA	EGFR	-0.51	-0.26	4.30E-01	8.29E-01
CCL8	CCR5	-0.49	0.71	4.52E-01	3.43E-01
	ACKR2*	-0.49	-2.98	4.52E-01	4.62E-03
	CCR2*	-0.49	1.97	4.52E-01	9.03E-03
IL16	CD4*	-0.47	3.45	4.71E-01	5.11E-03
CXCL10	CXCR3	-0.46	0.23	5.06E-01	
	DPP4*	-0.46	3.55	5.06E-01	1.16E-04
WNT5B	FZD6_LRP5	-0.45	0.33	5.19E-01	7.27E-01
	FZD6_LRP6	-0.45	0.33	5.19E-01	7.27E-01
	FZD8_LRP5	-0.45	-0.32	5.19E-01	7.36E-01
	FZD3_LRP5	-0.45	0.22	5.19E-01	8.35E-01
	FZD3_LRP6	-0.45	0.22	5.19E-01	8.35E-01
	FRZB*	-0.45	1.50	5.19E-01	5.79E-03
	FZD4_LRP5	-0.45	0.32	5.19E-01	7.50E-01
	FZD2_LRP5	-0.45	-0.29	5.19E-01	7.76E-01
	FZD8_LRP6	-0.45	-0.32	5.19E-01	7.36E-01
	FZD10_LRP5	-0.45	0.61	5.19E-01	3.02E-01
	FZD10_LRP6	-0.45	0.61	5.19E-01	3.02E-01
	FZD1_LRP6*	-0.45	2.46	5.19E-01	8.52E-07
	FZD2_LRP6	-0.45	-0.29	5.19E-01	7.76E-01
	FZD7_LRP5	-0.45	0.48	5.19E-01	5.46E-01
	SFRP1*	-0.45	5.64	5.19E-01	1.31E-11
	FZD7_LRP6	-0.45	0.48	5.19E-01	5.46E-01
	FZD1_LRP5*	-0.45	2.46	5.19E-01	8.52E-07
	SFRP5*	-0.45	5.71	5.19E-01	2.51E-05
	SFRP2	-0.45	0.19	5.19E-01	8.31E-01
	FZD4_LRP6	-0.45	0.32	5.19E-01	7.50E-01
	SFRP4*	-0.45	3.35	5.19E-01	1.64E-09
	FZD9_LRP5	-0.45	-0.07	5.19E-01	9.58E-01
	FZD5_LRP5	-0.45	-0.36	5.19E-01	6.95E-01
	FZD5_LRP6	-0.45	-0.36	5.19E-01	6.95E-01
	FZD9_LRP6	-0.45	-0.07	5.19E-01	9.58E-01
PDGFC	PDGFRA	-0.44	0.86	4.67E-01	2.56E-01
CCL28	CCR10	-0.44	-0.14	5.28E-01	9.00E-01
APLP2	PIGR	-0.40	0.25	4.89E-01	8.12E-01
	PLXNA4	-0.40	-0.25	4.89E-01	8.23E-01
IL36A	IL1RAP_IL1RL2	-0.38	-0.20	5.68E-01	8.60E-01
KISS1	KISS1R	-0.35	0.06	6.27E-01	9.76E-01
BTC	ERBB3	-0.30	1.13	6.83E-01	1.06E-01
	ERBB4	-0.30	-0.08	6.83E-01	9.60E-01
	EGFR	-0.30	-0.26	6.83E-01	8.29E-01

ligand	receptor	ligand_log2FC	receptor_log2FC	ligand_padj	receptor_padj
IHH	GAS1_PTCH1*	0.36	1.60	6.17E-01	8.97E-04
	BOC_PTCH1*	0.36	2.78	6.17E-01	1.60E-09
	CDON_PTCH1*	0.36	1.40	6.17E-01	4.19E-03
	HHIP	0.36	0.01	6.17E-01	9.93E-01
XCL1	XCR1	0.38	0.12	5.77E-01	
IL15_IL15RA	IL2RB_IL2RG	0.40	1.27	5.82E-01	8.32E-02
IL15	IL15RA_IL2RB*	0.40	1.40	5.82E-01	2.68E-03
SEMA3C	NRP2	0.40	0.27	4.70E-01	7.71E-01
PDGFA	PDGFRA	0.41	0.86	5.09E-01	2.56E-01
RSPO3	LGR6*	0.41	-6.16	5.41E-01	4.10E-15
	LGR4*	0.41	1.28	5.41E-01	6.28E-03
	LGR5*	0.41	2.98	5.41E-01	1.73E-04
PI16	TNFRSF21	0.44	0.41	5.12E-01	6.35E-01
IL7	IL2RG_IL7R	0.45	0.49	4.58E-01	5.45E-01
AGT	AGTR1	0.47	0.87	3.73E-01	1.45E-01
	AGTR2	0.47	-0.17	3.73E-01	8.94E-01
WNT7B	FZD2_LRP6	0.48	-0.29	4.66E-01	7.76E-01
	FZD10_LRP5	0.48	0.61	4.66E-01	3.02E-01
	FZD5_LRP5	0.48	-0.36	4.66E-01	6.95E-01
	SFRP2	0.48	0.19	4.66E-01	8.31E-01
	FZD1_LRP6*	0.48	2.46	4.66E-01	8.52E-07
	FZD10_LRP6	0.48	0.61	4.66E-01	3.02E-01
	FZD6_LRP6	0.48	0.33	4.66E-01	7.27E-01
	FZD6_LRP5	0.48	0.33	4.66E-01	7.27E-01
	FZD1_LRP5*	0.48	2.46	4.66E-01	8.52E-07
	FRZB*	0.48	1.50	4.66E-01	5.79E-03
	FZD2_LRP5	0.48	-0.29	4.66E-01	7.76E-01
	FZD8_LRP6	0.48	-0.32	4.66E-01	7.36E-01
	FZD3_LRP5	0.48	0.22	4.66E-01	8.35E-01
	FZD9_LRP6	0.48	-0.07	4.66E-01	9.58E-01
	SFRP1*	0.48	5.64	4.66E-01	1.31E-11
	FZD7_LRP5	0.48	0.48	4.66E-01	5.46E-01
	SFRP4*	0.48	3.35	4.66E-01	1.64E-09
	FZD3_LRP6	0.48	0.22	4.66E-01	8.35E-01
	FZD4_LRP5	0.48	0.32	4.66E-01	7.50E-01
	SFRP5*	0.48	5.71	4.66E-01	2.51E-05
	FZD7_LRP6	0.48	0.48	4.66E-01	5.46E-01
	FZD5_LRP6	0.48	-0.36	4.66E-01	6.95E-01
	FZD4_LRP6	0.48	0.32	4.66E-01	7.50E-01
	FZD8_LRP5	0.48	-0.32	4.66E-01	7.36E-01
	FZD9_LRP5	0.48	-0.07	4.66E-01	9.58E-01
APOE	TREM2_TYROBP*	0.51	-3.73	3.95E-01	2.34E-04
ANGPT1	TEK	0.52	-0.05	4.07E-01	9.75E-01
AREG	EGFR	0.52	-0.26	4.52E-01	8.29E-01
PSAP	GPR37	0.53	-0.85	3.13E-01	1.41E-01

ligand	receptor	ligand_log2FC	receptor_log2FC	ligand_padj	receptor_padj
RSPO1	LGR6*	0.54	-6.16	4.42E-01	4.10E-15
	LGR5*	0.54	2.98	4.42E-01	1.73E-04
	KREMEN1_LRP6	0.54	-0.32	4.42E-01	7.34E-01
	LGR4*	0.54	1.28	4.42E-01	6.28E-03
	FZD8_LRP6	0.54	-0.32	4.42E-01	7.36E-01
IL34	CSF1R	0.55	-0.04	4.36E-01	9.86E-01
ANGPT2	TEK	0.57	-0.05	3.82E-01	9.75E-01
LIF	IL6ST_LIFR*	0.59	-2.01	3.53E-01	7.37E-06
JAG1	VASN	0.60	-0.20	2.87E-01	8.51E-01
C4A	C5AR2	0.60	0.17	3.84E-01	
TGFB3	ACVR1_TGFBR2	0.61	-0.28	3.65E-01	7.89E-01
	ITGAV_ITGB6	0.61	-0.47	3.65E-01	4.89E-01
	TGFBR3	0.61	0.61	3.65E-01	6.02E-01
	ITGAV_ITGB8	0.61	-0.47	3.65E-01	4.89E-01
	TGFBR1_TGFBR2	0.61	-0.81	3.65E-01	1.00E-01
CSF1	CSF1R	0.65	-0.04	3.32E-01	9.86E-01
TGFB2	TGFBR1_TGFBR2	0.65	-0.81	1.06E-01	1.00E-01
	TGFBR3	0.65	0.61	1.06E-01	6.02E-01
	ACVR1_TGFBR2	0.65	-0.28	1.06E-01	7.89E-01
FGF9	FGFR3	0.69	-0.05	3.01E-01	9.77E-01
CCL4	ACKR2*	0.69	-2.98	3.22E-01	4.62E-03
	CCR5	0.69	0.71	3.22E-01	3.43E-01
TNFSF12	TNFRSF25	0.71	0.20	3.00E-01	8.64E-01
	TNFRSF12A	0.71	-0.70	3.00E-01	2.78E-01
HFE	TFRC	0.86	0.46	1.87E-01	5.67E-01
APELA	APLNR	0.87	0.85	1.32E-01	2.54E-01
ADM	RAMP3*	0.88	-6.36	9.18E-02	1.50E-43
	CALCRL_GPER1_RAMP3	0.88	-0.09	9.18E-02	9.70E-01
CCL25	ACKR4*	0.88	-2.45	1.59E-01	9.90E-05
	CCR9	0.88	0.01	1.59E-01	9.96E-01
WNT4	FZD4_LRP6	0.99	0.32	9.46E-02	7.50E-01
	FZD6_LRP5	0.99	0.33	9.46E-02	7.27E-01
	FZD5_LRP6	0.99	-0.36	9.46E-02	6.95E-01
	FZD9_LRP5	0.99	-0.07	9.46E-02	9.58E-01
	FZD7_LRP6	0.99	0.48	9.46E-02	5.46E-01
	FZD10_LRP6	0.99	0.61	9.46E-02	3.02E-01
	SFRP2	0.99	0.19	9.46E-02	8.31E-01
	FZD7_LRP5	0.99	0.48	9.46E-02	5.46E-01
	FZD8_LRP5	0.99	-0.32	9.46E-02	7.36E-01
	FZD8_LRP6	0.99	-0.32	9.46E-02	7.36E-01
	FZD9_LRP6	0.99	-0.07	9.46E-02	9.58E-01
	FZD1_LRP5*	0.99	2.46	9.46E-02	8.52E-07
	SFRP4*	0.99	3.35	9.46E-02	1.64E-09
	FZD3_LRP5	0.99	0.22	9.46E-02	8.35E-01
	FZD5_LRP5	0.99	-0.36	9.46E-02	6.95E-01

ligand	receptor	ligand_log2FC	receptor_log2FC	ligand_padj	receptor_padj
	FZD4_LRP5	0.99	0.32	9.46E-02	7.50E-01
	FZD2_LRP6	0.99	-0.29	9.46E-02	7.76E-01
	FZD2_LRP5	0.99	-0.29	9.46E-02	7.76E-01
	FZD3_LRP6	0.99	0.22	9.46E-02	8.35E-01
	SFRP5*	0.99	5.71	9.46E-02	2.51E-05
	SFRP1*	0.99	5.64	9.46E-02	1.31E-11
	FRZB*	0.99	1.50	9.46E-02	5.79E-03
	FZD10_LRP5	0.99	0.61	9.46E-02	3.02E-01
	FZD1_LRP6*	0.99	2.46	9.46E-02	8.52E-07
	FZD6_LRP6	0.99	0.33	9.46E-02	7.27E-01
HGF	MET*	1.05	-1.29	1.59E-01	3.71E-02
MPR1B_BMPR	BMPR1A	1.10	-0.41	5.12E-02	5.57E-01
RARRES2	CCRL2	1.17	-0.61	1.09E-01	3.70E-01
	CMKLR1	1.17	-0.97	1.09E-01	1.54E-01
	GPR1	1.17	-0.09	1.09E-01	9.45E-01
THPO	MPL	1.29	0.02	1.21E-01	
FTH1*	SCARA5*	1.48	1.49	2.10E-03	9.42E-04
CLU*	TREM2_TYROBP*	1.65	-3.73	2.10E-02	2.34E-04
GAS6*	AXL	1.70	-0.44	3.76E-03	4.20E-01
	TYRO3	1.70	0.17	3.76E-03	8.98E-01
	MERTK	1.70	-0.28	3.76E-03	8.41E-01
CXCL12*	CXCR4	1.75	0.17	2.73E-02	8.94E-01
	DPP4*	1.75	3.55	2.73E-02	1.16E-04
TF*	TFRC	2.34	0.46	6.74E-03	5.67E-01
	TFR2	2.34	0.46	6.74E-03	5.54E-01
CXCL14*	CXCR4	2.47	0.17	6.25E-05	8.94E-01
RBP4	STRA6	2.55	0.33	5.05E-02	7.39E-01
MMP2*	ITGAV_ITGB3	2.56	-0.47	9.12E-04	4.89E-01
VEGFD*	FLT4	2.60	0.12	2.75E-02	9.37E-01
	KDR*	2.60	-3.80	2.75E-02	9.91E-17
PGF*	FLT1	2.71	0.27	1.72E-03	8.01E-01
	NRP1	2.71	-0.23	1.72E-03	8.77E-01
	FLT1_KDR	2.71	0.27	1.72E-03	8.01E-01
	NRP2	2.71	0.27	1.72E-03	7.71E-01
GDF10*	ACVR1B_ACVR2B	2.91	0.22	1.74E-02	8.35E-01
FGL1*	LAG3	3.00	1.13	9.96E-04	7.11E-02
NMB*	NMBR	3.14	0.86	3.37E-04	2.27E-01
KNG1*	BDKRB2*	4.08	-1.20	3.38E-03	2.85E-02
	BDKRB1	4.08	0.15	3.38E-03	9.22E-01
PTPRD*	IL1RAP	4.47	-0.20	7.05E-25	8.60E-01
	IL1RAPL1	4.47	-0.11	7.05E-25	9.43E-01
CALCA*	CALCRL_RAMP1	4.82	-0.09	4.46E-05	9.70E-01
TAC3*	TACR3	6.24	0.24	4.36E-02	8.13E-01
	TACR1	6.24	0.45	4.36E-02	5.47E-01
IGFBP3*	TMEM219	6.45	0.00	4.65E-27	1.00E+00

ligand	receptor	ligand_log2FC	receptor_log2FC	ligand_padj	receptor_padj
NPY*	**NPY1R**	6.55	-0.25	3.21E-02	8.22E-01

Table S1.1. Differentially expressed ligand and receptor signaling from epithelial to stromal cells at implantation compared to diestrus in *Mus musculus*. * designates significant differences ($p < 0.05$)

ligand	receptor	ligand_log2FC	receptor_log2FC	ligand_padj	receptor_padj
KITLG	KIT*	-0.52	-2.71	3.44E-01	4.51E-15
JAG1	VASN	-0.49	0.25	2.58E-01	5.97E-01
SEMA3E	PLXND1	-0.24	1.03	5.92E-01	8.61E-02
NMB	GRPR	-0.11	-0.13		
FGF9	FGFR3	-0.11	-0.21		6.41E-01
IGFBP3	TMEM219	-0.10	0.10	6.77E-01	8.51E-01
SEMA3F	NRP2	0.11	0.67		1.66E-01
LTB	LTBR	0.11	0.30		5.05E-01
WNT5A	FZD4_LRP6	0.12	-0.06		9.14E-01
	FZD2_LRP6	0.12	0.06		9.17E-01
	FZD2_LRP5	0.12	0.06		9.17E-01
	FZD9_LRP5	0.12	0.03		
	FZD9_LRP6	0.12	0.03		
	SFRP2*	0.12	1.19		1.74E-03
	FZD3_LRP6	0.12	0.10		8.47E-01
	FZD10_LRP6*	0.12	3.02		3.97E-03
	FZD10_LRP5*	0.12	3.02		3.97E-03
	ROR1*	0.12	-0.82		1.37E-02
	FZD3_LRP5	0.12	0.10		8.47E-01
	FZD6_LRP5	0.12	0.06		
	FZD4_LRP5	0.12	-0.06		9.14E-01
	FZD6_LRP6	0.12	0.06		
	SFRP4*	0.12	1.55		6.45E-04
	FRZB	0.12	-0.05		9.25E-01
	SFRP1	0.12	0.43		2.31E-01
	ROR2	0.12	-0.59		7.04E-02
	WIF1	0.12	0.09		8.42E-01
WNT2	FZD9_LRP6	0.12	0.03		
	FZD9_LRP5	0.12	0.03		
	SFRP4*	0.12	1.55		6.45E-04
	FZD10_LRP6*	0.12	3.02		3.97E-03
	FZD2_LRP5	0.12	0.06		9.17E-01
	FZD2_LRP6	0.12	0.06		9.17E-01
	FRZB	0.12	-0.05		9.25E-01
	FZD4_LRP6	0.12	-0.06		9.14E-01
	FZD4_LRP5	0.12	-0.06		9.14E-01
	FZD3_LRP6	0.12	0.10		8.47E-01
	FZD3_LRP5	0.12	0.10		8.47E-01
	FZD6_LRP6	0.12	0.06		
	FZD6_LRP5	0.12	0.06		
	WIF1	0.12	0.09		8.42E-01
	FZD1_LRP6	0.12	0.12		
	FZD1_LRP5	0.12	0.12		
	SFRP2*	0.12	1.19		1.74E-03
	FZD10_LRP5*	0.12	3.02		3.97E-03
	SFRP1	0.12	0.43		2.31E-01

ligand	receptor	ligand_log2FC	receptor_log2FC	ligand_padj	receptor_padj
VEGFC	FLT4	0.13	0.40	7.91E-01	1.76E-01
	KDR*	0.13	4.68	7.91E-01	3.89E-09
WNT11	FZD4_LRP6	0.14	-0.06		9.14E-01
	FZD4_LRP5	0.14	-0.06		9.14E-01
	FZD3_LRP5	0.14	0.10		8.47E-01
	SFRP1	0.14	0.43		2.31E-01
	FZD9_LRP5	0.14	0.03		
	SFRP4*	0.14	1.55		6.45E-04
	FZD1_LRP6	0.14	0.12		
	FZD6_LRP6	0.14	0.06		
	FRZB	0.14	-0.05		9.25E-01
	SFRP2*	0.14	1.19		1.74E-03
	FZD3_LRP6	0.14	0.10		8.47E-01
	FZD9_LRP6	0.14	0.03		
	FZD10_LRP5*	0.14	3.02		3.97E-03
	FZD10_LRP6*	0.14	3.02		3.97E-03
	WIF1	0.14	0.09		8.42E-01
	FZD2_LRP6	0.14	0.06		9.17E-01
	FZD2_LRP5	0.14	0.06		9.17E-01
	FZD6_LRP5	0.14	0.06		
	FZD1_LRP5	0.14	0.12		
TGFB2	TGFBR3*	0.14	-0.85		5.25E-03
	TGFBR1_TGFBR2	0.14	-0.15		7.26E-01
	ACVR1_TGFBR2	0.14	0.05		9.27E-01
HDGF	NCL*	0.16	0.97	7.10E-01	3.14E-03
BMP6	ACVR1_ACVR2B	0.16	0.05		9.27E-01
	ACVR1_ACVR2A	0.16	0.05		9.27E-01
	ACVR1_BMPR2	0.16	0.05		9.27E-01
	BMPR1B_BMPR2	0.16	-0.11		7.94E-01
	ACVR2B_BMPR1A	0.16	-0.68		6.12E-02
	ACVR2B_BMPR1B	0.16	-0.68		6.12E-02
	BMPR1A_BMPR2	0.16	-0.08		8.73E-01
	ACVR2A_BMPR1B	0.16	-0.53		1.21E-01
	ACVR2A_BMPR1A	0.16	-0.53		1.21E-01
BMP5	ACVR2B_BMPR1A	0.19	-0.68		6.12E-02
	ACVR2A_BMPR1B	0.19	-0.53		1.21E-01
	ACVR2B_BMPR1B	0.19	-0.68		6.12E-02
	BMPR1B_BMPR2	0.19	-0.11		7.94E-01
	ACVR1_BMPR2	0.19	0.05		9.27E-01
	ACVR1_ACVR2B	0.19	0.05		9.27E-01
	ACVR1_ACVR2A	0.19	0.05		9.27E-01
	ACVR2A_BMPR1A	0.19	-0.53		1.21E-01
	BMPR1A_BMPR2	0.19	-0.08		8.73E-01
GPI	AMFR	0.19	-0.06	6.77E-01	9.05E-01
B2M_CD1B	IL6ST	0.23	-0.22	6.50E-01	5.54E-01

ligand	receptor	ligand_log2FC	receptor_log2FC	ligand_padj	receptor_padj
FTL	SCARA5	0.27	0.24	6.28E-01	5.92E-01
FTH1	SCARA5	0.28	0.24	5.83E-01	5.92E-01
LCN2	SLC22A17*	0.30	0.75	1.41E-01	2.68E-02
PSAP	GPR37L1	0.32	0.04	5.10E-01	
WNT7B	FZD9_LRP5	0.35	0.03	3.27E-01	
	FZD1_LRP5	0.35	0.12	3.27E-01	
	FZD2_LRP5	0.35	0.06	3.27E-01	9.17E-01
	FZD10_LRP6*	0.35	3.02	3.27E-01	3.97E-03
	FZD2_LRP6	0.35	0.06	3.27E-01	9.17E-01
	FZD6_LRP5	0.35	0.06	3.27E-01	
	FZD1_LRP6	0.35	0.12	3.27E-01	
	FZD3_LRP6	0.35	0.10	3.27E-01	8.47E-01
	FZD6_LRP6	0.35	0.06	3.27E-01	
	WIF1	0.35	0.09	3.27E-01	8.42E-01
	FZD3_LRP5	0.35	0.10	3.27E-01	8.47E-01
	FZD4_LRP5	0.35	-0.06	3.27E-01	9.14E-01
	FRZB	0.35	-0.05	3.27E-01	9.25E-01
	FZD4_LRP6	0.35	-0.06	3.27E-01	9.14E-01
	SFRP4*	0.35	1.55	3.27E-01	6.45E-04
	SFRP2*	0.35	1.19	3.27E-01	1.74E-03
	SFRP1	0.35	0.43	3.27E-01	2.31E-01
	FZD10_LRP5*	0.35	3.02	3.27E-01	3.97E-03
	FZD9_LRP6	0.35	0.03	3.27E-01	
VEGFB	NRP1	0.47	-0.51	3.57E-01	1.77E-01
	FLT1*	0.47	3.03	3.57E-01	5.88E-08
	FLT1_KDR*	0.47	3.03	3.57E-01	5.88E-08
TAX1BP3	ADGRB1	0.51	-0.30	2.39E-01	4.89E-01
	ADGRB2	0.51	0.07	2.39E-01	8.96E-01
PPIA*	BSG*	1.25	0.84	7.06E-03	1.56E-02
IHH	BOC_PTCH1	1.27	-0.07	6.19E-02	8.85E-01
	CDON_PTCH1	1.27	-0.50	6.19E-02	1.80E-01
	HHIP	1.27	0.53	6.19E-02	2.55E-01
VWF*	ITGAV_ITGB3	1.36	0.17	2.79E-02	6.95E-01
LGALS3*	MERTK	2.69	0.14	4.38E-12	7.85E-01
RARRES2	CMKLR1	3.09	-0.07		
APOA1	ABCA1*	4.30	-2.99		2.89E-22
	AMN_CUBN	4.30	0.09		
MDK	PTPRZ1	4.76	-0.08		8.83E-01
TF	TFRC*	5.24	0.85		2.97E-02

Table S1.2. Differentially expressed ligand and receptor signaling from epithelial to stromal cells at implantation compared to diestrus in *Cavia porcellus*. * designates significant differences ($p < 0.05$)

ligand	receptor	ligand_log2FC	receptor_log2FC	ligand_padj	receptor_padj
AREG*	EGFR*	-8.25	1.50	1.94E-52	2.91E-04
PTPRD*	IL1RAPL1*	-6.95	-2.11	7.96E-59	4.10E-02
	IL1RAP	-6.95	0.34	7.96E-59	6.07E-01
TNFSF15*	TNFRSF6B*	-6.91	2.32	2.32E-10	2.23E-04
WNT5A*	FZD6_LRP6	-6.11	0.81	6.96E-13	2.21E-01
	SFRP5	-6.11	-0.21	6.96E-13	7.83E-01
	FZD8_LRP5	-6.11	-0.13	6.96E-13	8.70E-01
	FZD4_LRP5	-6.11	-1.09	6.96E-13	1.58E-01
	FZD8_RYK	-6.11	-0.13	6.96E-13	8.70E-01
	FZD4_LRP6	-6.11	-1.09	6.96E-13	1.58E-01
	FZD6_LRP5	-6.11	0.81	6.96E-13	2.21E-01
	FZD8_LRP6	-6.11	-0.13	6.96E-13	8.70E-01
	FZD7_LRP5	-6.11	-0.18	6.96E-13	8.20E-01
	FZD7_LRP6	-6.11	-0.18	6.96E-13	8.20E-01
	FRZB*	-6.11	-4.84	6.96E-13	2.18E-13
	FZD2_LRP5	-6.11	-0.15	6.96E-13	8.38E-01
	ROR1	-6.11	0.68	6.96E-13	1.43E-01
	SFRP4*	-6.11	2.04	6.96E-13	8.19E-03
	FZD3_LRP6*	-6.11	-1.56	6.96E-13	6.48E-03
	SFRP1	-6.11	-0.07	6.96E-13	9.23E-01
	SFRP2*	-6.11	-6.05	6.96E-13	6.38E-34
	ROR2	-6.11	0.10	6.96E-13	8.84E-01
	FZD2_LRP6	-6.11	-0.15	6.96E-13	8.38E-01
	FZD3_LRP5*	-6.11	-1.56	6.96E-13	6.48E-03
	FZD10_LRP5*	-6.11	-1.79	6.96E-13	4.54E-03
	FZD10_LRP6*	-6.11	-1.79	6.96E-13	4.54E-03
WNT11*	FZD6_LRP5	-5.13	0.81	1.38E-23	2.21E-01
	FZD10_LRP6*	-5.13	-1.79	1.38E-23	4.54E-03
	FZD6_LRP6	-5.13	0.81	1.38E-23	2.21E-01
	FZD3_LRP5*	-5.13	-1.56	1.38E-23	6.48E-03
	FZD7_LRP5	-5.13	-0.18	1.38E-23	8.20E-01
	FRZB*	-5.13	-4.84	1.38E-23	2.18E-13
	SFRP1	-5.13	-0.07	1.38E-23	9.23E-01
	SFRP2*	-5.13	-6.05	1.38E-23	6.38E-34
	FZD1_LRP6	-5.13	-1.41	1.38E-23	6.48E-02
	FZD1_LRP5	-5.13	-1.41	1.38E-23	6.48E-02
	FZD8_LRP6	-5.13	-0.13	1.38E-23	8.70E-01
	FZD4_LRP5	-5.13	-1.09	1.38E-23	1.58E-01
	FZD2_LRP6	-5.13	-0.15	1.38E-23	8.38E-01
	FZD2_LRP5	-5.13	-0.15	1.38E-23	8.38E-01
	FZD4_LRP6	-5.13	-1.09	1.38E-23	1.58E-01
	FZD10_LRP5*	-5.13	-1.79	1.38E-23	4.54E-03
	FZD3_LRP6*	-5.13	-1.56	1.38E-23	6.48E-03
	FZD8_LRP5	-5.13	-0.13	1.38E-23	8.70E-01
	SFRP4*	-5.13	2.04	1.38E-23	8.19E-03
	FZD7_LRP6	-5.13	-0.18	1.38E-23	8.20E-01

ligand	receptor	ligand_log2FC	receptor_log2FC	ligand_padj	receptor_padj
	SFRP5	-5.13	-0.21	1.38E-23	7.83E-01
SOSTDC1*	LRP5	-5.05	0.41	1.41E-16	5.61E-01
	LRP6	-5.05	-0.38	1.41E-16	4.84E-01
NRTN*	GFRA2_RET*	-4.75	1.12	1.27E-14	4.63E-02
PDGFA*	PDGFRA*	-4.58	-1.09	7.03E-15	3.85E-02
IGFBP3*	TMEM219*	-4.42	1.14	1.32E-14	2.33E-02
WNT7A*	SFRP4*	-4.42	2.04	3.68E-11	8.19E-03
	SFRP1	-4.42	-0.07	3.68E-11	9.23E-01
	FZD2_LRP6	-4.42	-0.15	3.68E-11	8.38E-01
	FZD2_LRP5	-4.42	-0.15	3.68E-11	8.38E-01
	FZD4_LRP5	-4.42	-1.09	3.68E-11	1.58E-01
	SFRP2*	-4.42	-6.05	3.68E-11	6.38E-34
	FZD3_LRP6*	-4.42	-1.56	3.68E-11	6.48E-03
	FZD3_LRP5*	-4.42	-1.56	3.68E-11	6.48E-03
	FZD7_LRP6	-4.42	-0.18	3.68E-11	8.20E-01
	FZD8_LRP5	-4.42	-0.13	3.68E-11	8.70E-01
	FZD8_LRP6	-4.42	-0.13	3.68E-11	8.70E-01
	FZD10_LRP5*	-4.42	-1.79	3.68E-11	4.54E-03
	FZD4_LRP6	-4.42	-1.09	3.68E-11	1.58E-01
	FZD7_LRP5	-4.42	-0.18	3.68E-11	8.20E-01
	FZD6_LRP6	-4.42	0.81	3.68E-11	2.21E-01
	FZD1_LRP6	-4.42	-1.41	3.68E-11	6.48E-02
	FZD10_LRP6*	-4.42	-1.79	3.68E-11	4.54E-03
	FZD6_LRP5	-4.42	0.81	3.68E-11	2.21E-01
	SFRP5	-4.42	-0.21	3.68E-11	7.83E-01
	FZD1_LRP5	-4.42	-1.41	3.68E-11	6.48E-02
	FRZB*	-4.42	-4.84	3.68E-11	2.18E-13
EDN1*	EDNRB	-4.23	-0.62	1.09E-16	3.32E-01
	EDNRA*	-4.23	-1.38	1.09E-16	6.51E-03
CSF1*	CSF1R	-4.20	0.30	1.36E-17	6.96E-01
SEMA3F*	NRP2	-4.09	-0.11	3.76E-14	8.87E-01
BMP4*	ACVR2A_BMPR1B	-3.97	0.39	1.50E-08	4.91E-01
	BMPR1B_BMPR2*	-3.97	1.73	1.50E-08	4.90E-03
	BMPR1A_BMPR2	-3.97	0.10	1.50E-08	8.99E-01
	ACVR2B_BMPR1B*	-3.97	-1.95	1.50E-08	3.10E-05
	ACVR2B_BMPR1A*	-3.97	-1.95	1.50E-08	3.10E-05
	ACVR2A_BMPR1A	-3.97	0.39	1.50E-08	4.91E-01
LGALS3*	MERTK*	-3.90	1.50	1.72E-13	4.05E-04
GAS6*	AXL	-3.82	0.18	2.23E-08	7.95E-01
	TYRO3	-3.82	0.20	2.23E-08	8.03E-01
	MERTK*	-3.82	1.50	2.23E-08	4.05E-04
FGF9*	FGFR3	-3.81	0.22	5.89E-11	7.63E-01
IL34*	CSF1R	-3.74	0.30	3.15E-04	6.96E-01
TGFB2*	TGFBR1_TGFBR2*	-3.62	-1.14	2.90E-13	1.20E-02
	ACVR1_TGFBR2	-3.62	0.15	2.90E-13	8.28E-01

ligand	receptor	ligand_log2FC	receptor_log2FC	ligand_padj	receptor_padj
	TGFBR3*	-3.62	1.39	2.90E-13	6.99E-04
EREG*	EGFR*	-3.56	1.50	5.60E-11	2.91E-04
	ERBB4*	-3.56	1.72	5.60E-11	2.60E-03
PDGFC*	PDGFRA*	-3.50	-1.09	3.47E-12	3.85E-02
HBEGF*	EGFR*	-3.12	1.50	1.17E-05	2.91E-04
	ERBB4*	-3.12	1.72	1.17E-05	2.60E-03
	ERBB2	-3.12	0.04	1.17E-05	9.60E-01
EDN2*	EDNRA*	-3.03	-1.38	1.80E-07	6.51E-03
	EDNRB	-3.03	-0.62	1.80E-07	3.32E-01
LIF*	IL6ST_LIFR	-2.95	0.33	5.12E-06	5.69E-01
ANXA1*	FPR2	-2.86	0.20	6.62E-09	7.72E-01
EDN3*	EDNRA*	-2.84	-1.38	1.28E-03	6.51E-03
	EDNRB	-2.84	-0.62	1.28E-03	3.32E-01
ANGPT1*	TEK*	-2.76	2.97	2.69E-07	3.60E-05
TNFSF9*	TNFRSF9	-2.61	0.81	1.98E-02	2.52E-01
CCL28*	CCR10	-2.48	-0.13	1.86E-03	8.47E-01
PTN*	PTPRZ1	-2.28	-0.19	1.96E-03	7.85E-01
	ALK*	-2.28	2.53	1.96E-03	7.91E-04
C3*	C5AR2	-2.24	0.59	5.25E-04	4.01E-01
	ITGAM_ITGB2	-2.24	0.15	5.25E-04	
SEMA3C*	NRP2	-2.12	-0.11	9.29E-04	8.87E-01
IL16*	CD4	-2.08	-0.25	4.56E-04	7.33E-01
APP*	FPR2	-2.03	0.20	1.85E-05	7.72E-01
	SORL1	-2.03	-0.11	1.85E-05	8.78E-01
	TNFRSF21	-2.03	-0.02	1.85E-05	9.87E-01
PTHLH*	PTH1R	-1.72	0.68	1.12E-02	1.96E-01
	PTH2R	-1.72	0.72	1.12E-02	2.94E-01
GDNF*	GFRA1_RET*	-1.70	-2.38	4.68E-02	7.41E-07
FGF10*	FGFR2	-1.69	-0.11	1.14E-02	8.81E-01
RSPO3*	LGR6	-1.65	-0.19	3.17E-02	7.69E-01
	LGR5	-1.65	-1.26	3.17E-02	8.73E-02
	LGR4	-1.65	0.21	3.17E-02	7.27E-01
WNT7B*	FZD1_LRP6	-1.51	-1.41	2.23E-02	6.48E-02
	FZD10_LRP6*	-1.51	-1.79	2.23E-02	4.54E-03
	FZD6_LRP6	-1.51	0.81	2.23E-02	2.21E-01
	FZD10_LRP5*	-1.51	-1.79	2.23E-02	4.54E-03
	SFRP1	-1.51	-0.07	2.23E-02	9.23E-01
	SFRP4*	-1.51	2.04	2.23E-02	8.19E-03
	FZD8_LRP6	-1.51	-0.13	2.23E-02	8.70E-01
	FZD8_LRP5	-1.51	-0.13	2.23E-02	8.70E-01
	SFRP2*	-1.51	-6.05	2.23E-02	6.38E-34
	FZD3_LRP6*	-1.51	-1.56	2.23E-02	6.48E-03
	FZD7_LRP5	-1.51	-0.18	2.23E-02	8.20E-01
	FZD1_LRP5	-1.51	-1.41	2.23E-02	6.48E-02
	FZD4_LRP6	-1.51	-1.09	2.23E-02	1.58E-01

ligand	receptor	ligand_log2FC	receptor_log2FC	ligand_padj	receptor_padj
	FZD4_LRP5	-1.51	-1.09	2.23E-02	1.58E-01
	FZD2_LRP6	-1.51	-0.15	2.23E-02	8.38E-01
	FZD2_LRP5	-1.51	-0.15	2.23E-02	8.38E-01
	SFRP5	-1.51	-0.21	2.23E-02	7.83E-01
	FZD3_LRP5*	-1.51	-1.56	2.23E-02	6.48E-03
	FZD6_LRP5	-1.51	0.81	2.23E-02	2.21E-01
	FZD7_LRP6	-1.51	-0.18	2.23E-02	8.20E-01
	FRZB*	-1.51	-4.84	2.23E-02	2.18E-13
C4A*	C5AR2	-1.15	0.59	2.16E-02	4.01E-01
HDGF*	NCL	-1.09	-0.78	1.98E-02	9.66E-02
BMPR1B_BMPR2*	BMPR1A	-0.95	0.10	4.97E-02	8.99E-01
VEGFC*	KDR	1.51	1.43	3.02E-02	8.39E-02
	FLT4*	1.51	2.58	3.02E-02	3.99E-03
BMP8A*	ACVR2B_BMPR1A*	1.59	-1.95	3.13E-02	3.10E-05
	BMPR1B_BMPR2*	1.59	1.73	3.13E-02	4.90E-03
	ACVR2A_BMPR1B	1.59	0.39	3.13E-02	4.91E-01
	ACVR1_BMPR2	1.59	0.15	3.13E-02	8.28E-01
	ACVR2B_BMPR1B*	1.59	-1.95	3.13E-02	3.10E-05
	ACVR2A_BMPR1A	1.59	0.39	3.13E-02	4.91E-01
	ACVR1_ACVR2B	1.59	0.15	3.13E-02	8.28E-01
	BMPR1A_BMPR2	1.59	0.10	3.13E-02	8.99E-01
	ACVR1_ACVR2A	1.59	0.15	3.13E-02	8.28E-01
REN*	ATP6AP2	1.86	0.66	6.19E-03	1.71E-01
LCN2*	SLC22A17	2.25	-0.26	6.39E-03	7.02E-01
IL15*	IL15RA_IL2RB	2.28	-0.23	2.05E-04	7.58E-01
IL15_IL15RA*	IL2RB_IL2RG	2.28	0.24	2.05E-04	7.30E-01
CCL19*	CCR7	2.43	0.21	4.78E-02	
	ACKR4	2.43	1.06	4.78E-02	1.64E-01
	CCRL2*	2.43	2.11	4.78E-02	2.83E-05
TNFSF13B*	TNFRSF13B	2.48	0.06	8.47E-04	9.35E-01
IHH*	GAS1_PTCH1	2.83	-0.01	8.67E-07	9.94E-01
	CDON_PTCH1	2.83	-0.62	8.67E-07	3.28E-01
	HHIP	2.83	-0.84	8.67E-07	1.33E-01
AVP*	OXTR	3.28	-0.27	4.13E-03	6.52E-01
	AVPR1A	3.28	-0.40	4.13E-03	5.75E-01
CXCL8*	ACKR1	3.28	0.03	3.67E-02	9.73E-01
CCL21*	CCR7	3.36	0.21	3.11E-04	
	ACKR4	3.36	1.06	3.11E-04	1.64E-01
RBP4*	STRA6*	3.68	2.83	3.82E-08	1.88E-07
BMP2*	ACVR2B_BMPR1B*	4.79	-1.95	4.90E-21	3.10E-05
	ACVR2B_BMPR1A*	4.79	-1.95	4.90E-21	3.10E-05
	BMPR1B_BMPR2*	4.79	1.73	4.90E-21	4.90E-03
	ACVR2A_BMPR1B	4.79	0.39	4.90E-21	4.91E-01
	ACVR2A_BMPR1A	4.79	0.39	4.90E-21	4.91E-01
	BMPR1A_BMPR2	4.79	0.10	4.90E-21	8.99E-01

ligand	receptor	ligand_log2FC	receptor_log2FC	ligand_padj	receptor_padj
NTF3*	**NTRK2**	6.14	0.86	2.78E-30	5.70E-02
	NGFR*	6.14	2.79	2.78E-30	3.70E-11
	NTRK3*	6.14	-3.01	2.78E-30	1.34E-08
IGF1*	**IGFBP2***	7.02	5.04	6.75E-40	1.78E-21
	IGFBP1	7.02	-0.30	6.75E-40	6.82E-01
	IGF1R	7.02	-0.37	6.75E-40	5.03E-01
	IGFBP3*	7.02	-1.93	6.75E-40	1.41E-30
	ITGAV_ITGB3	7.02	0.54	6.75E-40	2.67E-01
	IGFBP4	7.02	0.67	6.75E-40	2.33E-01
	GPC3_IGF1R	7.02	-0.27	6.75E-40	7.09E-01
	ITGA6_ITGB4	7.02	1.06	6.75E-40	1.32E-01
	IGFBP5*	7.02	-1.86	6.75E-40	4.70E-03
MSTN*	**ACVR2B_TGFBR1***	11.68	-1.95	2.12E-05	3.10E-05
	ACVR2A_TGFBR1	11.68	0.39	2.12E-05	4.91E-01

Table S1.3. Differentially expressed ligand and receptor signaling from epithelial to stromal cells at day 7.5 compared to the non-pregnant *Monodelphis domestica*. * designates significant differences ($p < 0.05$)

ligand	receptor	ligand_log2FC	receptor_log2FC	ligand_padj	receptor_padj
NMB*	GRPR	-8.27	-0.26	8.35E-35	6.80E-01
PDGFC*	PDGFRA	-7.15	0.17	1.18E-38	8.52E-01
WNT7A*	SFRP2*	-7.10	-6.31	4.39E-08	7.05E-22
	FZD7_LRP5	-7.10	-0.02	4.39E-08	9.79E-01
	FZD1_LRP5	-7.10	-0.44	4.39E-08	5.72E-01
	FZD4_LRP6	-7.10	-0.21	4.39E-08	7.69E-01
	FZD1_LRP6	-7.10	-0.44	4.39E-08	5.72E-01
	FZD7_LRP6	-7.10	-0.02	4.39E-08	9.79E-01
	FZD3_LRP6	-7.10	-1.46	4.39E-08	7.26E-02
	FZD6_LRP5	-7.10	0.01	4.39E-08	9.95E-01
	SFRP1	-7.10	-0.08	4.39E-08	8.89E-01
	SFRP4	-7.10	-0.22	4.39E-08	6.88E-01
	FZD2_LRP5	-7.10	0.77	4.39E-08	3.12E-01
	FZD8_LRP6	-7.10	-0.74	4.39E-08	3.68E-01
	FZD10_LRP6	-7.10	-0.33	4.39E-08	6.88E-01
	SFRP5	-7.10	-0.26	4.39E-08	
	FZD6_LRP6	-7.10	0.01	4.39E-08	9.95E-01
	FZD2_LRP6	-7.10	0.77	4.39E-08	3.12E-01
	FZD8_LRP5	-7.10	-0.74	4.39E-08	3.68E-01
	FZD3_LRP5	-7.10	-1.46	4.39E-08	7.26E-02
	FRZB*	-7.10	-2.63	4.39E-08	1.07E-03
	FZD10_LRP5	-7.10	-0.33	4.39E-08	6.88E-01
	FZD4_LRP5	-7.10	-0.21	4.39E-08	7.69E-01
IHH*	GAS1_PTCH1	-5.78	-0.43	1.01E-03	5.06E-01
	HHIP*	-5.78	-4.94	1.01E-03	7.69E-06
	CDON_PTCH1	-5.78	0.62	1.01E-03	4.35E-01
TGM2*	ADGRG1	-4.77	0.00	1.36E-17	
PTN*	PTPRZ1*	-4.61	2.63	2.87E-06	1.13E-06
	ALK	-4.61	0.16	2.87E-06	
ANGPT1*	TEK	-4.57	0.25	2.26E-11	7.36E-01
PDGFA*	PDGFRA	-4.11	0.17	3.64E-15	8.52E-01
PTHLH*	PTH2R	-3.85	0.21	2.96E-04	8.22E-01
	PTH1R	-3.85	-0.50	2.96E-04	5.30E-01
	PRLHR	-3.85	-0.06	2.96E-04	
BMP4*	ACVR2B_BMPR1B	-3.67	-0.16	3.10E-05	8.66E-01
	ACVR2A_BMPR1B*	-3.67	1.56	3.10E-05	2.86E-03
	BMPR1B_BMPR2*	-3.67	2.80	3.10E-05	3.64E-04
	BMPR1A_BMPR2	-3.67	-0.06	3.10E-05	9.52E-01
	ACVR2A_BMPR1A*	-3.67	1.56	3.10E-05	2.86E-03
	ACVR2B_BMPR1A	-3.67	-0.16	3.10E-05	8.66E-01
WNT5A*	FZD8_LRP5	-3.55	-0.74	2.01E-04	3.68E-01
	FZD2_LRP6	-3.55	0.77	2.01E-04	3.12E-01
	FZD2_LRP5	-3.55	0.77	2.01E-04	3.12E-01
	FZD8_LRP6	-3.55	-0.74	2.01E-04	3.68E-01
	SFRP2*	-3.55	-6.31	2.01E-04	7.05E-22
	FZD7_LRP6	-3.55	-0.02	2.01E-04	9.79E-01

ligand	receptor	ligand_log2FC	receptor_log2FC	ligand_padj	receptor_padj
	FZD7_LRP5	-3.55	-0.02	2.01E-04	9.79E-01
	FZD3_LRP5	-3.55	-1.46	2.01E-04	7.26E-02
	FZD8_RYK	-3.55	-0.74	2.01E-04	3.68E-01
	SFRP5	-3.55	-0.26	2.01E-04	
	FZD3_LRP6	-3.55	-1.46	2.01E-04	7.26E-02
	FRZB*	-3.55	-2.63	2.01E-04	1.07E-03
	FZD10_LRP5	-3.55	-0.33	2.01E-04	6.88E-01
	SFRP4	-3.55	-0.22	2.01E-04	6.88E-01
	FZD10_LRP6	-3.55	-0.33	2.01E-04	6.88E-01
	ROR1	-3.55	0.19	2.01E-04	8.23E-01
	FZD6_LRP5	-3.55	0.01	2.01E-04	9.95E-01
	FZD6_LRP6	-3.55	0.01	2.01E-04	9.95E-01
	FZD4_LRP5	-3.55	-0.21	2.01E-04	7.69E-01
	FZD4_LRP6	-3.55	-0.21	2.01E-04	7.69E-01
	SFRP1	-3.55	-0.08	2.01E-04	8.89E-01
	ROR2	-3.55	-0.30	2.01E-04	7.08E-01
EDN2*	EDNRB	-3.55	-0.53	2.15E-07	5.07E-01
	EDNRA	-3.55	-0.09	2.15E-07	9.27E-01
WNT5B*	SFRP4	-3.51	-0.22	3.50E-09	6.88E-01
	FZD10_LRP5	-3.51	-0.33	3.50E-09	6.88E-01
	FZD7_LRP5	-3.51	-0.02	3.50E-09	9.79E-01
	FZD4_LRP6	-3.51	-0.21	3.50E-09	7.69E-01
	FZD6_LRP5	-3.51	0.01	3.50E-09	9.95E-01
	FZD8_LRP5	-3.51	-0.74	3.50E-09	3.68E-01
	FRZB*	-3.51	-2.63	3.50E-09	1.07E-03
	FZD8_LRP6	-3.51	-0.74	3.50E-09	3.68E-01
	FZD6_LRP6	-3.51	0.01	3.50E-09	9.95E-01
	FZD4_LRP5	-3.51	-0.21	3.50E-09	7.69E-01
	FZD3_LRP5	-3.51	-1.46	3.50E-09	7.26E-02
	FZD3_LRP6	-3.51	-1.46	3.50E-09	7.26E-02
	FZD1_LRP6	-3.51	-0.44	3.50E-09	5.72E-01
	SFRP2*	-3.51	-6.31	3.50E-09	7.05E-22
	FZD2_LRP6	-3.51	0.77	3.50E-09	3.12E-01
	SFRP5	-3.51	-0.26	3.50E-09	
	FZD7_LRP6	-3.51	-0.02	3.50E-09	9.79E-01
	FZD1_LRP5	-3.51	-0.44	3.50E-09	5.72E-01
	SFRP1	-3.51	-0.08	3.50E-09	8.89E-01
	FZD2_LRP5	-3.51	0.77	3.50E-09	3.12E-01
	FZD10_LRP6	-3.51	-0.33	3.50E-09	6.88E-01
EDN3*	EDNRA	-3.50	-0.09	8.43E-03	9.27E-01
	EDNRB	-3.50	-0.53	8.43E-03	5.07E-01
AREG*	EGFR	-3.49	0.74	9.44E-06	1.71E-01
JAG2*	VASN	-3.25	-0.40	4.04E-11	5.66E-01
SEMA3F*	NRP2	-3.01	1.28	1.55E-05	5.57E-02
GDNF*	GFRA1_RET*	-3.00	-1.65	4.19E-02	4.44E-03

ligand	receptor	ligand_log2FC	receptor_log2FC	ligand_padj	receptor_padj
SEMA3A*	NRP1_PLXNA3	-3.00	-0.79	8.14E-06	1.95E-01
	NRP1_PLXNA1	-3.00	-0.79	8.14E-06	1.95E-01
	NRP1_PLXNA2	-3.00	-0.79	8.14E-06	1.95E-01
	NRP1	-3.00	-0.79	8.14E-06	1.95E-01
RARRES2*	CCRL2*	-2.98	2.22	1.27E-04	4.51E-04
	GPR1	-2.98	0.56	1.27E-04	5.04E-01
	CMKLR1	-2.98	-0.33	1.27E-04	5.79E-01
RSPO3*	LGR5	-2.89	-6.54	1.54E-02	1.40E-01
	LGR4	-2.89	-0.08	1.54E-02	9.36E-01
	LGR6	-2.89	0.23	1.54E-02	7.29E-01
EDN1*	EDNRB	-2.88	-0.53	2.01E-05	5.07E-01
	EDNRA	-2.88	-0.09	2.01E-05	9.27E-01
PTPRD*	IL1RAP*	-2.79	2.03	7.38E-09	1.95E-03
	IL1RAPL1	-2.79	-0.57	7.38E-09	4.92E-01
CXCL12*	DPP4	-2.78	0.47	2.05E-02	5.13E-01
	CXCR4	-2.78	0.30	2.05E-02	6.64E-01
IL16*	CD4	-2.72	-0.23	6.75E-04	6.83E-01
BMPR1B_BMPR2*	BMPR1A	-2.68	-0.06	9.52E-10	9.52E-01
AGRN*	LRP4_MUSK	-2.65	0.42	2.61E-06	5.92E-01
	PTPRS*	-2.65	-2.72	2.61E-06	4.06E-05
MMP2*	ITGAV_ITGB3	-2.64	0.31	3.09E-05	6.79E-01
EREG*	EGFR	-2.45	0.74	5.82E-04	1.71E-01
	ERBB4*	-2.45	1.84	5.82E-04	2.19E-02
LGALS3*	MERTK	-2.21	0.76	5.51E-06	2.38E-01
TGFB2*	TGFBR1_TGFBR2	-2.20	-0.72	1.12E-04	2.80E-01
	TGFBR3	-2.20	0.58	1.12E-04	3.74E-01
	ACVR1_TGFBR2	-2.20	0.14	1.12E-04	8.74E-01
REN*	ATP6AP2	-2.16	-0.08	2.05E-02	9.32E-01
PDGFD*	PDGFRB	-2.12	1.05	2.76E-02	8.48E-02
	PDGFRA_PDGFRB	-2.12	0.17	2.76E-02	8.52E-01
KITLG*	KIT	-2.08	-0.18	2.50E-02	
ANXA1*	FPR2	-1.90	-0.11	1.52E-03	
GAS6*	MERTK	-1.67	0.76	2.24E-02	2.38E-01
	TYRO3	-1.67	-0.02	2.24E-02	9.75E-01
	AXL	-1.67	-0.28	2.24E-02	7.38E-01
WNT11*	FZD2_LRP5	-1.23	0.77	3.47E-02	3.12E-01
	FZD6_LRP5	-1.23	0.01	3.47E-02	9.95E-01
	FZD6_LRP6	-1.23	0.01	3.47E-02	9.95E-01
	FZD1_LRP5	-1.23	-0.44	3.47E-02	5.72E-01
	SFRP4	-1.23	-0.22	3.47E-02	6.88E-01
	FZD8_LRP6	-1.23	-0.74	3.47E-02	3.68E-01
	FZD8_LRP5	-1.23	-0.74	3.47E-02	3.68E-01
	SFRP5	-1.23	-0.26	3.47E-02	
	SFRP1	-1.23	-0.08	3.47E-02	8.89E-01
	FRZB*	-1.23	-2.63	3.47E-02	1.07E-03

ligand	receptor	ligand_log2FC	receptor_log2FC	ligand_padj	receptor_padj
	FZD3_LRP6	-1.23	-1.46	3.47E-02	7.26E-02
	FZD3_LRP5	-1.23	-1.46	3.47E-02	7.26E-02
	SFRP2*	-1.23	-6.31	3.47E-02	7.05E-22
	FZD7_LRP5	-1.23	-0.02	3.47E-02	9.79E-01
	FZD7_LRP6	-1.23	-0.02	3.47E-02	9.79E-01
	FZD10_LRP5	-1.23	-0.33	3.47E-02	6.88E-01
	FZD1_LRP6	-1.23	-0.44	3.47E-02	5.72E-01
	FZD10_LRP6	-1.23	-0.33	3.47E-02	6.88E-01
	FZD4_LRP6	-1.23	-0.21	3.47E-02	7.69E-01
	FZD4_LRP5	-1.23	-0.21	3.47E-02	7.69E-01
	FZD2_LRP6	-1.23	0.77	3.47E-02	3.12E-01
RNASET2*	TLR8	-1.21	-0.42	1.44E-02	5.95E-01
APP*	FPR2	-1.10	-0.11	1.94E-02	
	TNFRSF21	-1.10	0.21	1.94E-02	8.26E-01
	SORL1	-1.10	-0.14	1.94E-02	8.76E-01
HDGF*	NCL	-1.08	-0.74	2.59E-02	2.19E-01
FTL*	SCARA5	-1.04	0.65	3.26E-02	3.08E-01
BMP2*	ACVR2B_BMPR1B	1.44	-0.16	4.32E-02	8.66E-01
	BMPR1B_BMPR2*	1.44	2.80	4.32E-02	3.64E-04
	ACVR2B_BMPR1A	1.44	-0.16	4.32E-02	8.66E-01
	ACVR2A_BMPR1A*	1.44	1.56	4.32E-02	2.86E-03
	BMPR1A_BMPR2	1.44	-0.06	4.32E-02	9.52E-01
	ACVR2A_BMPR1B*	1.44	1.56	4.32E-02	2.86E-03
JAG1*	VASN	1.47	-0.40	2.27E-02	5.66E-01
ADM2*	CALCRL_RAMP2	1.64	-0.30	2.00E-02	7.01E-01
TNFSF13B*	TNFRSF13B	2.18	0.31	1.43E-02	
LCN2*	SLC22A17	2.23	-0.15	1.52E-02	
CXCL2*	DPP4	2.60	0.47	6.96E-05	5.13E-01
BMP8A*	ACVR1_BMPR2	2.74	0.14	4.79E-04	8.74E-01
	ACVR2A_BMPR1B*	2.74	1.56	4.79E-04	2.86E-03
	BMPR1A_BMPR2	2.74	-0.06	4.79E-04	9.52E-01
	ACVR1_ACVR2B	2.74	0.14	4.79E-04	8.74E-01
	ACVR2B_BMPR1A	2.74	-0.16	4.79E-04	8.66E-01
	BMPR1B_BMPR2*	2.74	2.80	4.79E-04	3.64E-04
	ACVR1_ACVR2A	2.74	0.14	4.79E-04	8.74E-01
	ACVR2A_BMPR1A*	2.74	1.56	4.79E-04	2.86E-03
	ACVR2B_BMPR1B	2.74	-0.16	4.79E-04	8.66E-01
FGF1*	ITGAV_ITGB3	2.82	0.31	1.18E-04	6.79E-01
	FGFR1	2.82	-0.12	1.18E-04	9.03E-01
	FGFR4	2.82	-0.02	1.18E-04	
	FGFR3	2.82	-0.18	1.18E-04	
	FGFR2*	2.82	-1.88	1.18E-04	9.99E-04
NTF3*	NGFR	2.93	1.11	6.93E-08	9.91E-02
	NTRK2	2.93	-0.26	6.93E-08	7.81E-01
	NTRK3	2.93	-1.33	6.93E-08	5.20E-02

ligand	receptor	ligand_log2FC	receptor_log2FC	ligand_padj	receptor_padj
IGFBP3*	**TMEM219**	3.01	0.11	1.02E-08	9.08E-01
GDF15*	**ERBB2**	3.38	-0.25	9.18E-06	7.69E-01
BDNF*	**SORT1**	3.91	-0.18	5.23E-05	8.52E-01
	NTRK2	3.91	-0.26	5.23E-05	7.81E-01
	NGFR	3.91	1.11	5.23E-05	9.91E-02
CXCL9*	**CXCR3**	4.09	-0.07	7.51E-04	
	DPP4	4.09	0.47	7.51E-04	5.13E-01
FGL1*	**LAG3**	4.81	0.24	1.26E-08	7.90E-01
APOB*	**APOBR**	5.31	0.29	4.94E-06	
CXCL8*	**ACKR1***	5.87	3.47	6.06E-04	7.05E-04
APOA1*	**AMN_CUBN**	6.45	-0.37	1.24E-04	5.83E-01
	ABCA1*	6.45	1.75	1.24E-04	9.86E-04
ANGPT2*	**TEK**	6.92	0.25	2.82E-30	7.36E-01
RBP4*	**STRA6**	11.92	-0.22	6.45E-100	8.12E-01

Table S1.4. Differentially expressed ligand and receptor signaling from epithelial to stromal cells at day 13.5 compared to the non-pregnant *Monodelphis domestica*. * designates significant differences ($p < 0.05$)

source	target	ligand_complex	receptor_complex	cellphone_pvals	specificity_rank	magnitude_rank	ligand_%_exp	receptor_%_exp
ESF	LE	ADM	RAMP3	0.341	0.375	0.860	0.21	0.27
	LE	ANGPT1	TEK	0.444	0.436	0.528	0.38	0.20
	LE	APLP2	PIGR	0.000	0.001	0.029	0.87	0.67
	LE	APP	SORL1	0.000	0.001	0.002	0.97	0.86
	LE	APP	TNFRSF21	0.000	0.001	0.002	0.97	0.92
	LE	BMP7	ACVR1_ACVR2A	0.007	0.017	0.344	0.21	0.65
	LE	BMP7	ACVR1_ACVR2B	0.007	0.017	0.344	0.21	0.41
	LE	BMP7	ACVR1_BMPR2	0.007	0.017	0.344	0.21	0.82
	LE	BMP7	ACVR2A_BMPR1A	0.416	0.055	0.468	0.21	0.65
	LE	BMP7	ACVR2A_BMPR1B	0.416	0.055	0.468	0.21	0.65
	LE	BMP7	ACVR2B_BMPR1A	0.000	0.002	0.763	0.21	0.41
	LE	BMP7	ACVR2B_BMPR1B	0.000	0.002	0.763	0.21	0.41
	LE	BMP7	BMPR1A_BMPR2	0.178	0.038	0.135	0.21	0.86
	LE	BMP7	BMPR1B_BMPR2	0.000	0.001	0.551	0.21	0.37
	LE	CXCL12	DPP4	0.000	0.001	0.010	0.87	0.71
	LE	FGF10	FGFR2	0.000	0.000	0.004	0.26	1.00
	LE	FGF2	FGFR2	0.000	0.001	0.004	0.32	1.00
	LE	FTH1	SCARA5	1.000	1.000	0.017	1.00	0.20
	LE	FTL	SCARA5	1.000	1.000	0.024	1.00	0.20
	LE	GAS6	AXL	1.000	1.000	0.381	0.54	0.29
	LE	GAS6	TYRO3	0.767	0.270	0.381	0.54	0.47
	LE	GDF10	ACVR1B_ACVR2B	0.000	0.001	0.179	0.61	0.41
	LE	GDF11	ACVR1B_ACVR2A	0.000	0.002	0.546	0.25	0.67
	LE	GDF11	ACVR1B_ACVR2B	0.000	0.002	0.546	0.25	0.41
	LE	GDF11	ACVR2A_TGFBR1	0.646	0.313	0.466	0.25	0.65
	LE	GDF11	ACVR2B_TGFBR1	0.001	0.013	0.760	0.25	0.41
	LE	GPI	AMFR	0.400	0.316	0.092	0.81	0.71
	LE	HDGF	NCL	0.010	0.018	0.001	0.79	0.96
	LE	HFE	TFRC	0.000	0.001	0.041	0.83	0.57
	LE	HGF	MET	0.000	0.001	0.100	0.58	0.80
	LE	HSD17B12	AR	1.000	1.000	0.421	0.54	0.29
	LE	IGF1	GPC3_IGF1R	0.000	0.004	0.006	1.00	0.24
	LE	IGF1	IGF1R	0.000	0.001	0.000	1.00	0.98
	LE	IGF1	IGFBP3	0.000	0.000	0.000	1.00	0.90
	LE	IGF1	IGFBP4	0.625	1.000	0.002	1.00	0.47
	LE	IGF1	IGFBP5	0.020	0.021	0.002	1.00	0.41
	LE	IGF1	IGFBP6	0.432	0.780	0.003	1.00	0.37
	LE	IGF1	ITGA6_ITGB4	0.000	0.001	0.000	1.00	0.88
	LE	IGFBP3	TMEM219	0.000	0.003	0.022	0.78	0.51
	LE	LIPA	RORA	1.000	1.000	0.066	0.46	0.86
	LE	LIPA	RORC	0.001	0.013	0.504	0.46	0.45
	LE	PDGFC	PDGFRA	1.000	1.000	0.046	0.81	0.22
	LE	PI16	TNFRSF21	0.000	0.001	0.050	0.37	0.92
	LE	PPIA	BSG	0.525	0.524	0.000	1.00	0.98
	LE	PTGES	PTGER2	0.000	0.000	0.096	0.35	0.80
	LE	PTGES	PTGER4	0.096	0.031	0.369	0.35	0.67
	LE	PTGES3	PTGER2	0.000	0.001	0.015	0.83	0.80
	LE	PTGES3	PTGER4	0.894	0.952	0.055	0.83	0.67
	LE	PTPRD	IL1RAP	0.056	0.041	0.162	0.65	0.31
	LE	RBP4	STRA6	0.406	0.054	0.849	0.25	0.27
	LE	RSPO1	FZD8_LRP6	0.030	0.023	0.882	0.23	0.22
	LE	RSPO1	KREMEN1_LRP6	0.000	0.001	0.236	0.23	0.84
	LE	RSPO1	LGR4	0.001	0.013	0.359	0.23	0.67
	LE	RSPO1	LGR5	0.000	0.000	0.101	0.23	0.67
	LE	RSPO3	LGR4	0.027	0.262	0.254	0.32	0.67
	LE	RSPO3	LGR5	0.000	0.000	0.060	0.32	0.67
	LE	SRD5A3	AR	1.000	1.000	0.756	0.29	0.29

source	target	ligand_complex	receptor_complex	cellphone_pvals	specificity_rank	magnitude_rank	ligand_%_exp	receptor_%_exp
	LE	SULT1A1	PPARD	0.952	0.333	0.530	0.47	0.29
	LE	TF	TFRC	0.000	0.090	0.466	0.22	0.57
	LE	TGFB2	ACVR1_TGFBR2	0.015	0.020	0.098	0.63	0.82
	LE	TGFB2	TGFBR1_TGFBR2	0.519	0.521	0.108	0.63	0.78
	LE	TGFB2	TGFBR3	1.000	1.000	0.108	0.63	0.76
	LE	TGFB3	ACVR1_TGFBR2	0.025	0.022	0.316	0.28	0.82
	LE	TGFB3	ITGAV_ITGB6	0.060	0.028	0.107	0.28	0.27
	LE	TGFB3	ITGAV_ITGB8	0.060	0.028	0.107	0.28	0.69
	LE	TGFB3	TGFBR1_TGFBR2	0.592	0.560	0.361	0.28	0.78
	LE	TGFB3	TGFBR3	1.000	1.000	0.362	0.28	0.76
	LE	TGM2	ADGRG1	0.000	0.002	0.103	0.69	0.69
	LE	TNFSF12	TNFRSF12A	0.342	0.188	0.506	0.51	0.33
	LE	WNT5A	FZD10_LRP5	0.000	0.001	0.133	0.83	0.29
	LE	WNT5A	FZD10_LRP6	0.000	0.001	0.133	0.83	0.29
	LE	WNT5A	FZD3_LRP5	0.000	0.001	0.060	0.83	0.49
	LE	WNT5A	FZD3_LRP6	0.000	0.001	0.060	0.83	0.61
	LE	WNT5A	FZD5_LRP5	0.000	0.001	0.095	0.83	0.49
	LE	WNT5A	FZD5_LRP6	0.000	0.001	0.095	0.83	0.51
	LE	WNT5A	FZD6_LRP5	0.000	0.001	0.028	0.83	0.49
	LE	WNT5A	FZD6_LRP6	0.000	0.001	0.028	0.83	0.86
	LE	WNT5A	FZD8_LRP5	0.000	0.001	0.131	0.83	0.22
	LE	WNT5A	FZD8_LRP6	0.000	0.001	0.131	0.83	0.22
	LE	WNT5A	FZD8_RYK	0.000	0.001	0.131	0.83	0.22
	LE	WNT5A	ROR2	0.088	0.031	0.011	0.83	0.86
	LE	WNT5A	SFRP1	0.229	0.041	0.129	0.83	0.27
	LE	WNT5A	SFRP4	0.990	1.000	0.125	0.83	0.35
	LE	WNT5B	FZD10_LRP5	0.000	0.001	0.394	0.50	0.29
	LE	WNT5B	FZD10_LRP6	0.000	0.001	0.394	0.50	0.29
	LE	WNT5B	FZD1_LRP5	0.999	1.000	0.403	0.50	0.22
	LE	WNT5B	FZD1_LRP6	0.999	1.000	0.403	0.50	0.22
	LE	WNT5B	FZD3_LRP5	0.000	0.001	0.205	0.50	0.49
	LE	WNT5B	FZD3_LRP6	0.000	0.001	0.205	0.50	0.61
	LE	WNT5B	FZD5_LRP5	0.000	0.001	0.269	0.50	0.49
	LE	WNT5B	FZD5_LRP6	0.000	0.001	0.269	0.50	0.51
	LE	WNT5B	FZD6_LRP5	0.000	0.001	0.103	0.50	0.49
	LE	WNT5B	FZD6_LRP6	0.000	0.001	0.103	0.50	0.86
	LE	WNT5B	FZD8_LRP5	0.000	0.001	0.390	0.50	0.22
	LE	WNT5B	FZD8_LRP6	0.000	0.001	0.390	0.50	0.22
	LE	WNT5B	SFRP1	0.767	0.582	0.381	0.50	0.27
	LE	WNT5B	SFRP4	1.000	1.000	0.370	0.50	0.35
	MPH	APLP2	PLXNA4	0.000	0.010	0.032	0.87	0.33
	MPH	APOE	TREM2_TYROBP	0.000	0.001	0.006	0.89	0.43
	MPH	APP	PLXNA4	0.000	0.005	0.006	0.97	0.33
	MPH	APP	SORL1	0.000	0.003	0.006	0.97	0.44
	MPH	APP	TNFRSF21	0.000	0.005	0.007	0.97	0.41
	MPH	APP	TREM2_TYROBP	0.000	0.001	0.004	0.97	0.43
	MPH	BMP7	ACVR1_ACVR2A	0.989	0.189	0.551	0.21	0.30
	MPH	BMP7	ACVR1_BMPR2	0.989	0.189	0.551	0.21	0.30
	MPH	CSF1	CSF1R	0.000	0.000	0.020	0.30	0.73
	MPH	CXCL12	CXCR4	0.000	0.000	0.013	0.87	0.43
	MPH	CXCL12	DPP4	0.000	0.001	0.024	0.87	0.25
	MPH	CXCL14	CXCR4	0.000	0.000	0.062	0.48	0.43
	MPH	DPEP1	CYSLTR1	0.000	0.001	0.143	0.46	0.47
	MPH	FGF10	FGFR2	1.000	1.000	0.457	0.26	0.25
	MPH	FGF2	FGFR2	1.000	1.000	0.467	0.32	0.25
	MPH	GAS6	AXL	1.000	1.000	0.109	0.54	0.45

source	target	ligand_complex	receptor_complex	cellphone_pvals	specificity_rank	magnitude_rank	ligand_%_exp	receptor_%_exp
	MPH	GAS6	MERTK	0.989	0.189	0.108	0.54	0.38
	MPH	GDF11	ACVR2A_TGFBR1	1.000	1.000	0.537	0.25	0.26
	MPH	GPI	AMFR	0.028	0.026	0.076	0.81	0.41
	MPH	HDGF	NCL	1.000	1.000	0.007	0.79	0.78
	MPH	IGF1	IGF1R	0.000	0.019	0.000	1.00	0.43
	MPH	IGF1	IGFBP4	0.000	0.023	0.000	1.00	0.48
	MPH	IGFBP3	TMEM219	0.000	0.001	0.016	0.78	0.31
	MPH	LGALS3	MERTK	0.998	0.656	0.225	0.25	0.38
	MPH	LIPA	RORA	1.000	1.000	0.410	0.46	0.24
	MPH	PI16	TNFRSF21	0.000	0.001	0.261	0.37	0.41
	MPH	PPIA	BSG	1.000	1.000	0.000	1.00	0.72
	MPH	PTGES	PTGER4	0.000	0.001	0.169	0.35	0.47
	MPH	PTGES3	PTGER4	0.000	0.003	0.026	0.83	0.47
	MPH	RARRES2	CCRL2	0.000	0.001	0.016	0.92	0.36
	MPH	RARRES2	CMKLR1	0.000	0.017	0.029	0.92	0.32
	MPH	RNASET2	TLR8	0.000	0.001	0.491	0.21	0.34
	MPH	SEMA3A	NRP1	0.000	0.001	0.010	0.46	0.67
	MPH	SEMA3A	NRP1_PLXNA4	0.000	0.001	0.010	0.46	0.33
	MPH	SEMA3C	NRP2	0.000	0.003	0.021	0.86	0.31
	MPH	SULT1A1	PPARD	0.000	0.001	0.153	0.47	0.50
	MPH	SULT1A1	PPARG	0.000	0.001	0.214	0.47	0.32
	MPH	TGFB2	ACVR1_TGFBR2	1.000	1.000	0.169	0.63	0.30
	MPH	TGFB2	TGFBR1_TGFBR2	0.000	0.001	0.029	0.63	0.56
	MPH	TGFB3	ACVR1_TGFBR2	1.000	1.000	0.524	0.28	0.30
	MPH	TGFB3	TGFBR1_TGFBR2	0.000	0.001	0.120	0.28	0.56
	MPH	TNFSF13B	TNFRSF13B	0.000	0.001	0.459	0.25	0.33
	MPH	VEGFA	NRP1	0.000	0.001	0.021	0.47	0.67
	MPH	VEGFA	NRP2	0.410	0.054	0.260	0.47	0.31
	MPH	VEGFB	NRP1	0.000	0.001	0.022	0.48	0.67
LE	ESF	AGRN	PTPRS	0.000	0.001	0.098	0.73	0.78
	ESF	ALDH1A2	RORB	0.000	0.006	0.027	0.24	0.97
	ESF	APP	TNFRSF21	0.935	1.000	0.020	0.98	0.21
	ESF	AREG	EGFR	0.000	0.001	0.009	0.35	0.95
	ESF	BMPR1B_BMPR2	BMPR1A	0.000	0.001	0.053	0.37	0.89
	ESF	BTC	EGFR	0.000	0.001	0.023	0.33	0.95
	ESF	DHCR24	RORA	0.000	0.001	0.001	0.55	0.99
	ESF	DHCR7	NR1H2	0.002	0.014	0.614	0.47	0.31
	ESF	DHCR7	NR1H3	0.000	0.002	0.583	0.47	0.32
	ESF	DHCR7	RORA	0.000	0.001	0.003	0.47	0.99
	ESF	EDN1	EDNRA	0.000	0.000	0.041	0.53	0.67
	ESF	EDN1	EDNRB	0.000	0.000	0.101	0.53	0.36
	ESF	FTH1	SCARA5	0.000	0.001	0.000	1.00	0.83
	ESF	FTL	SCARA5	0.360	0.057	0.000	0.94	0.83
	ESF	GAS6	AXL	1.000	1.000	0.199	0.29	0.81
	ESF	GAS6	MERTK	1.000	1.000	0.188	0.29	0.74
	ESF	GLS_SLC1A1	GRIA3	0.382	0.461	0.044	0.90	0.57
	ESF	GLS_SLC1A1	GRIA4	0.000	0.002	0.009	0.90	0.70
	ESF	GLS_SLC1A1	GRIK2	0.004	0.016	0.051	0.90	0.49
	ESF	GLS_SLC1A1	GRIK2_GRIK5	0.004	0.016	0.051	0.90	0.30
	ESF	GLS_SLC1A1	GRM7	0.999	1.000	0.153	0.90	0.21
	ESF	GPI	AMFR	0.914	0.909	0.122	0.86	0.46
	ESF	HBEGF	EGFR	0.000	0.000	0.003	0.67	0.95
	ESF	HDGF	NCL	0.000	0.001	0.000	0.98	0.96
	ESF	HSD17B12	AR	0.000	0.001	0.004	0.84	0.97
	ESF	IGF1	GPC3_IGF1R	1.000	1.000	0.315	0.27	0.59
	ESF	IGF1	IGF1R	1.000	1.000	0.022	0.27	0.94

source	target	ligand_complex	receptor_complex	cellphone_pvals	specificity_rank	magnitude_rank	ligand_%_exp	receptor_%_exp
	ESF	IGF1	IGFBP3	1.000	1.000	0.025	0.27	0.78
	ESF	IGF1	IGFBP4	1.000	1.000	0.015	0.27	0.97
	ESF	IGF1	IGFBP5	1.000	1.000	0.058	0.27	0.71
	ESF	IGF1	IGFBP6	1.000	1.000	0.018	0.27	0.94
	ESF	IGF1	ITGAV_ITGB3	1.000	1.000	0.232	0.27	0.43
	ESF	IGFBP3	TMEM219	0.000	0.001	0.005	0.90	0.31
	ESF	IHH	BOC_PTCH1	0.000	0.001	0.129	0.61	0.50
	ESF	IHH	CDON_PTCH1	0.000	0.001	0.210	0.61	0.29
	ESF	IHH	GAS1_PTCH1	0.000	0.001	0.018	0.61	0.86
	ESF	IL1A	IL1R1_IL1RAP	0.000	0.002	0.255	0.29	0.36
	ESF	IL36A	IL1RAP_IL1RL2	0.000	0.001	0.473	0.27	0.36
	ESF	IL36B	IL1RAP_IL1RL2	0.000	0.001	0.640	0.31	0.36
	ESF	KNG1	BDKRB2	0.000	0.001	0.426	0.31	0.41
	ESF	LCN2	SLC22A17	0.000	0.001	0.392	0.57	0.23
	ESF	LGALS3	MERTK	0.000	0.001	0.044	0.76	0.74
	ESF	LIPA	RORA	0.000	0.001	0.000	0.69	0.99
	ESF	MMP2	ITGAV_ITGB3	1.000	1.000	0.192	0.43	0.43
	ESF	PDGFA	PDGFRA	0.000	0.000	0.001	0.86	0.99
	ESF	PDGFC	PDGFRA	0.120	0.344	0.004	0.49	0.99
	ESF	PDGFD	PDGFRA_PDGFRB	0.017	0.020	0.010	0.31	0.59
	ESF	PDGFD	PDGFRB	1.000	1.000	0.384	0.31	0.59
	ESF	PGF	NRP1	0.000	0.001	0.032	0.53	0.74
	ESF	PGF	NRP2	0.000	0.003	0.192	0.53	0.29
	ESF	PPIA	BSG	0.620	0.857	0.000	0.98	0.93
	ESF	PTGES2	PTGER4	0.000	0.003	0.378	0.37	0.57
	ESF	PTGES3	PTGER4	0.000	0.002	0.020	0.94	0.57
	ESF	RARRES2	CMKLR1	1.000	1.000	0.777	0.24	0.25
	ESF	SEMA3C	NRP2	0.001	0.013	0.010	0.94	0.29
	ESF	SEMA3F	NRP2	1.000	1.000	0.779	0.20	0.29
	ESF	SRD5A1	AR	0.000	0.000	0.001	0.90	0.97
	ESF	SRD5A3	AR	0.000	0.001	0.012	0.49	0.97
	ESF	TGFA	EGFR	0.000	0.001	0.008	0.55	0.95
	ESF	TGFB1	ACVR1_TGFBR2	1.000	1.000	0.498	0.20	0.54
	ESF	TGFB1	TGFBR1_TGFBR2	1.000	1.000	0.404	0.20	0.60
	ESF	TGFB1	TGFBR3	0.000	0.083	0.026	0.20	0.99
	ESF	TGFB2	ACVR1_TGFBR2	1.000	1.000	0.431	0.20	0.54
	ESF	TGFB2	TGFBR1_TGFBR2	1.000	1.000	0.360	0.20	0.60
	ESF	TGFB2	TGFBR3	0.012	0.170	0.009	0.20	0.99
	ESF	UBASH3B	ESR1	0.000	0.001	0.000	0.84	1.00
	ESF	VEGFA	NRP1	0.329	0.439	0.060	0.63	0.74
	ESF	VEGFA	NRP2	0.996	1.000	0.376	0.63	0.29
	ESF	VEGFB	NRP1	1.000	1.000	0.153	0.29	0.74
	ESF	WNT11	FRZB	0.000	0.001	0.174	0.71	0.25
	ESF	WNT11	FZD1_LRP5	0.000	0.000	0.041	0.71	0.34
	ESF	WNT11	FZD1_LRP6	0.000	0.000	0.041	0.71	0.72
	ESF	WNT11	FZD2_LRP5	0.000	0.000	0.041	0.71	0.34
	ESF	WNT11	FZD2_LRP6	0.000	0.000	0.041	0.71	0.75
	ESF	WNT11	FZD3_LRP5	0.000	0.001	0.163	0.71	0.32
	ESF	WNT11	FZD3_LRP6	0.000	0.001	0.163	0.71	0.32
	ESF	WNT11	SFRP1	0.000	0.001	0.107	0.71	0.29
	ESF	WNT11	SFRP2	0.000	0.000	0.035	0.71	0.68
	ESF	WNT11	SFRP4	0.000	0.000	0.011	0.71	0.68
	ESF	WNT5A	FRZB	0.024	0.022	0.142	0.90	0.25
	ESF	WNT5A	FZD2_LRP5	0.000	0.001	0.031	0.90	0.34
	ESF	WNT5A	FZD2_LRP6	0.000	0.001	0.031	0.90	0.75
	ESF	WNT5A	FZD3_LRP5	0.001	0.013	0.130	0.90	0.32

source	target	ligand_complex	receptor_complex	cellphone_pvals	specificity_rank	magnitude_rank	ligand_%_exp	receptor_%_exp
	ESF	WNT5A	FZD3_LRP6	0.001	0.013	0.130	0.90	0.32
	ESF	WNT5A	ROR1	0.000	0.001	0.005	0.90	0.85
	ESF	WNT5A	ROR2	0.000	0.001	0.003	0.90	0.95
	ESF	WNT5A	SFRP1	0.010	0.018	0.092	0.90	0.29
	ESF	WNT5A	SFRP2	0.000	0.001	0.028	0.90	0.68
	ESF	WNT5A	SFRP4	0.000	0.001	0.009	0.90	0.68
	ESF	WNT7A	FRZB	0.000	0.001	0.278	0.82	0.25
	ESF	WNT7A	FZD1_LRP5	0.000	0.000	0.059	0.82	0.34
	ESF	WNT7A	FZD1_LRP6	0.000	0.000	0.059	0.82	0.72
	ESF	WNT7A	FZD2_LRP5	0.000	0.000	0.060	0.82	0.34
	ESF	WNT7A	FZD2_LRP6	0.000	0.000	0.060	0.82	0.75
	ESF	WNT7A	FZD3_LRP5	0.000	0.001	0.247	0.82	0.32
	ESF	WNT7A	FZD3_LRP6	0.000	0.001	0.247	0.82	0.32
	ESF	WNT7A	SFRP1	0.000	0.000	0.161	0.82	0.29
	ESF	WNT7A	SFRP2	0.000	0.000	0.053	0.82	0.68
	ESF	WNT7A	SFRP4	0.000	0.000	0.019	0.82	0.68
	ESF	WNT7B	FRZB	0.000	0.000	0.027	0.82	0.25
	ESF	WNT7B	FZD1_LRP5	0.000	0.000	0.007	0.82	0.34
	ESF	WNT7B	FZD1_LRP6	0.000	0.000	0.007	0.82	0.72
	ESF	WNT7B	FZD2_LRP5	0.000	0.000	0.007	0.82	0.34
	ESF	WNT7B	FZD2_LRP6	0.000	0.000	0.007	0.82	0.75
	ESF	WNT7B	FZD3_LRP5	0.000	0.000	0.026	0.82	0.32
	ESF	WNT7B	FZD3_LRP6	0.000	0.000	0.026	0.82	0.32
	ESF	WNT7B	SFRP1	0.000	0.000	0.018	0.82	0.29
	ESF	WNT7B	SFRP2	0.000	0.000	0.006	0.82	0.68
	ESF	WNT7B	SFRP4	0.000	0.000	0.002	0.82	0.68
	VEC	AGRN	PTPRS	1.000	1.000	0.413	0.73	0.29
	VEC	ANGPT2	TEK	0.000	0.000	0.010	0.41	0.92
	VEC	APLP2	PLXNA4	0.000	0.001	0.003	0.94	0.74
	VEC	APP	PLXNA4	0.000	0.001	0.001	0.98	0.74
	VEC	APP	TNFRSF21	0.967	1.000	0.022	0.98	0.21
	VEC	DHCR24	RORA	0.294	0.170	0.003	0.55	0.90
	VEC	DHCR7	NR1H2	0.000	0.003	0.545	0.47	0.38
	VEC	DHCR7	RORA	0.546	0.277	0.006	0.47	0.90
	VEC	GAS6	MERTK	1.000	1.000	0.369	0.29	0.56
	VEC	GPI	AMFR	0.488	0.155	0.095	0.86	0.54
	VEC	HDGF	NCL	0.000	0.002	0.000	0.98	0.89
	VEC	HSD17B12	AR	1.000	1.000	0.146	0.84	0.37
	VEC	IGF1	IGF1R	1.000	1.000	0.060	0.27	0.78
	VEC	IGF1	IGFBP3	1.000	1.000	0.264	0.27	0.48
	VEC	IGF1	IGFBP4	1.000	1.000	0.028	0.27	0.84
	VEC	IGF1	IGFBP6	1.000	1.000	0.816	0.27	0.21
	VEC	IGF1	ITGA6_ITGB4	1.000	1.000	0.018	0.27	0.62
	VEC	IGF1	ITGAV_ITGB3	1.000	1.000	0.558	0.27	0.40
	VEC	IGFBP3	TMEM219	0.000	0.001	0.006	0.90	0.30
	VEC	IHH	GAS1_PTCH1	1.000	0.057	0.245	0.61	0.24
	VEC	IL1A	IL1R1_IL1RAP	0.000	0.001	0.199	0.29	0.25
	VEC	KITLG	KIT	0.330	0.239	0.504	0.20	0.36
	VEC	KNG1	BDKRB2	0.000	0.003	0.640	0.31	0.23
	VEC	LGALS3	MERTK	0.011	0.019	0.098	0.76	0.56
	VEC	LIPA	RORA	0.044	0.025	0.001	0.69	0.90
	VEC	MMP2	ITGAV_ITGB3	1.000	1.000	0.482	0.43	0.40
	VEC	PGF	FLT1	0.000	0.000	0.000	0.53	0.98
	VEC	PGF	FLT1_KDR	0.000	0.000	0.000	0.53	0.93
	VEC	PGF	NRP1	0.000	0.000	0.003	0.53	0.91
	VEC	PGF	NRP2	0.000	0.000	0.005	0.53	0.85

source	target	ligand_complex	receptor_complex	cellphone_pvals	specificity_rank	magnitude_rank	ligand_%_exp	receptor_%_exp
	VEC	PPIA	BSG	0.002	0.014	0.000	0.98	0.91
	VEC	SEMA3C	NRP2	0.000	0.000	0.000	0.94	0.85
	VEC	SEMA3E	PLXND1	0.000	0.000	0.002	0.71	0.92
	VEC	SEMA3F	NRP2	0.000	0.008	0.055	0.20	0.85
	VEC	SRD5A1	AR	0.006	0.017	0.038	0.90	0.37
	VEC	SRD5A3	AR	1.000	1.000	0.421	0.49	0.37
	VEC	TGFB1	ACVR1_TGFBR2	0.998	1.000	0.490	0.20	0.50
	VEC	TGFB1	TGFBR1_TGFBR2	1.000	1.000	0.472	0.20	0.52
	VEC	TGFB1	TGFBR3	1.000	1.000	0.042	0.20	0.86
	VEC	TGFB2	ACVR1_TGFBR2	1.000	1.000	0.426	0.20	0.50
	VEC	TGFB2	TGFBR1_TGFBR2	1.000	1.000	0.414	0.20	0.52
	VEC	TGFB2	TGFBR3	1.000	1.000	0.018	0.20	0.86
	VEC	TGM2	ADGRG1	0.007	0.017	0.136	0.69	0.58
	VEC	TNFSF10	TNFRSF10A	0.000	0.001	0.484	0.39	0.40
	VEC	TNFSF10	TNFRSF11B	0.000	0.001	0.487	0.39	0.32
	VEC	TNFSF12	TNFRSF12A	1.000	1.000	0.955	0.31	0.21
	VEC	UBASH3B	ESR1	1.000	1.000	0.061	0.84	0.48
	VEC	VEGFA	FLT1	0.000	0.000	0.001	0.63	0.98
	VEC	VEGFA	FLT1_KDR	0.000	0.000	0.001	0.63	0.93
	VEC	VEGFA	KDR	0.000	0.000	0.005	0.63	0.93
	VEC	VEGFA	NRP1	0.000	0.001	0.005	0.63	0.91
	VEC	VEGFA	NRP2	0.000	0.001	0.009	0.63	0.85
	VEC	VEGFB	FLT1	0.000	0.001	0.013	0.29	0.98
	VEC	VEGFB	FLT1_KDR	0.000	0.001	0.013	0.29	0.93
	VEC	VEGFB	NRP1	0.000	0.004	0.023	0.29	0.91
	VEC	WNT11	FZD4_LRP5	0.000	0.000	0.032	0.71	0.61
	VEC	WNT11	FZD4_LRP6	0.000	0.000	0.032	0.71	0.66
	VEC	WNT11	FZD6_LRP5	0.000	0.000	0.025	0.71	0.61
	VEC	WNT11	FZD6_LRP6	0.000	0.000	0.025	0.71	0.66
	VEC	WNT5A	FZD4_LRP5	0.000	0.001	0.027	0.90	0.61
	VEC	WNT5A	FZD4_LRP6	0.000	0.001	0.027	0.90	0.66
	VEC	WNT5A	FZD6_LRP5	0.000	0.001	0.021	0.90	0.61
	VEC	WNT5A	FZD6_LRP6	0.000	0.001	0.021	0.90	0.66
	VEC	WNT7A	FZD4_LRP5	0.000	0.000	0.051	0.82	0.61
	VEC	WNT7A	FZD4_LRP6	0.000	0.000	0.051	0.82	0.66
	VEC	WNT7A	FZD6_LRP5	0.000	0.000	0.038	0.82	0.61
	VEC	WNT7A	FZD6_LRP6	0.000	0.000	0.038	0.82	0.66
	VEC	WNT7B	FZD4_LRP5	0.000	0.000	0.006	0.82	0.61
	VEC	WNT7B	FZD4_LRP6	0.000	0.000	0.006	0.82	0.66
	VEC	WNT7B	FZD6_LRP5	0.000	0.000	0.004	0.82	0.61
	VEC	WNT7B	FZD6_LRP6	0.000	0.000	0.004	0.82	0.66
MPH	ESF	APP	TNFRSF21	0.000	0.023	0.011	0.88	0.21
	ESF	FTH1	SCARA5	0.000	0.001	0.000	0.98	0.83
	ESF	FTL	SCARA5	0.000	0.001	0.000	0.97	0.83
	ESF	GAS6	AXL	0.000	0.005	0.034	0.35	0.81
	ESF	GAS6	MERTK	0.000	0.002	0.032	0.35	0.74
	ESF	GPI	AMFR	0.512	0.078	0.095	0.64	0.46
	ESF	HDGF	NCL	1.000	1.000	0.003	0.53	0.96
	ESF	HSD17B12	AR	0.000	0.001	0.002	0.62	0.97
	ESF	IGF1	GPC3_IGF1R	1.000	1.000	0.063	0.41	0.59
	ESF	IGF1	IGF1R	1.000	1.000	0.005	0.41	0.94
	ESF	IGF1	IGFBP3	1.000	1.000	0.006	0.41	0.78
	ESF	IGF1	IGFBP4	1.000	1.000	0.002	0.41	0.97
	ESF	IGF1	IGFBP5	1.000	1.000	0.016	0.41	0.71
	ESF	IGF1	IGFBP6	1.000	1.000	0.003	0.41	0.94
	ESF	IGF1	ITGAV_ITGB3	1.000	1.000	0.049	0.41	0.43

source	target	ligand_complex	receptor_complex	cellphone_pvals	specificity_rank	magnitude_rank	ligand_%_exp	receptor_%_exp
	ESF	IL1B	IL1R1_IL1RAP	0.000	0.001	0.015	0.43	0.36
	ESF	LGALS3	MERTK	0.000	0.001	0.004	0.70	0.74
	ESF	LIPA	RORA	0.000	0.001	0.000	0.53	0.99
	ESF	OSM	IL6ST_OSMR	0.692	0.089	0.164	0.24	0.21
	ESF	PDGFB	PDGFRA	0.000	0.001	0.002	0.33	0.99
	ESF	PDGFB	PDGFRA_PDGFRB	0.000	0.001	0.002	0.33	0.59
	ESF	PDGFB	PDGFRB	0.000	0.003	0.172	0.33	0.59
	ESF	PDGFC	PDGFRA	0.000	0.010	0.003	0.23	0.99
	ESF	PPIA	BSG	1.000	1.000	0.000	0.96	0.93
	ESF	PTGES3	PTGER4	0.026	0.022	0.040	0.68	0.57
	ESF	TGFB1	ACVR1_TGFBR2	0.000	0.001	0.031	0.72	0.54
	ESF	TGFB1	TGFBR1_TGFBR2	0.000	0.001	0.025	0.72	0.60
	ESF	TGFB1	TGFBR3	0.000	0.001	0.000	0.72	0.99
	ESF	TNF	TNFRSF1A	0.000	0.001	0.156	0.31	0.65
	ESF	UBASH3B	ESR1	0.000	0.001	0.000	0.74	1.00
	VEC	APLP2	PLXNA4	0.000	0.001	0.006	0.63	0.74
	VEC	APP	PLXNA4	0.000	0.001	0.000	0.88	0.74
	VEC	APP	TNFRSF21	0.000	0.036	0.016	0.88	0.21
	VEC	GAS6	MERTK	0.002	0.018	0.060	0.35	0.56
	VEC	GPI	AMFR	0.000	0.011	0.057	0.64	0.54
	VEC	HDGF	NCL	1.000	1.000	0.004	0.53	0.89
	VEC	HSD17B12	AR	1.000	1.000	0.090	0.62	0.37
	VEC	IGF1	IGF1R	1.000	1.000	0.016	0.41	0.78
	VEC	IGF1	IGFBP3	1.000	1.000	0.054	0.41	0.48
	VEC	IGF1	IGFBP4	1.000	1.000	0.007	0.41	0.84
	VEC	IGF1	IGFBP6	1.000	1.000	0.220	0.41	0.21
	VEC	IGF1	ITGA6_ITGB4	0.965	0.377	0.003	0.41	0.62
	VEC	IGF1	ITGAV_ITGB3	1.000	1.000	0.150	0.41	0.40
	VEC	IL1B	IL1R1_IL1RAP	0.000	0.000	0.010	0.43	0.25
	VEC	KITLG	KIT	0.000	0.002	0.275	0.26	0.36
	VEC	LGALS3	MERTK	0.000	0.001	0.007	0.70	0.56
	VEC	LIPA	RORA	0.000	0.001	0.001	0.53	0.90
	VEC	OSM	IL6ST_LIFR	0.000	0.000	0.006	0.24	0.57
	VEC	OSM	IL6ST_OSMR	0.000	0.000	0.006	0.24	0.59
	VEC	PLAU	PLAUR	0.000	0.001	0.297	0.28	0.38
	VEC	PPIA	BSG	0.000	0.006	0.000	0.96	0.91
	VEC	TGFB1	ACVR1_TGFBR2	0.000	0.001	0.030	0.72	0.50
	VEC	TGFB1	TGFBR1_TGFBR2	0.000	0.001	0.029	0.72	0.52
	VEC	TGFB1	TGFBR3	0.000	0.001	0.001	0.72	0.86
	VEC	TGM2	ADGRG1	0.000	0.001	0.122	0.37	0.58
	VEC	TNF	TNFRSF1A	0.000	0.000	0.032	0.31	0.81
	VEC	TNFSF12	TNFRSF12A	0.498	0.061	0.533	0.31	0.21
	VEC	TNFSF9	TNFRSF9	0.000	0.000	0.160	0.28	0.45
	VEC	UBASH3B	ESR1	1.000	1.000	0.011	0.74	0.48
VEC	LE	AGRN	PTPRS	0.968	0.654	0.368	0.47	0.65
	LE	ANGPT2	TEK	0.062	0.028	0.627	0.30	0.20
	LE	APLP2	PIGR	0.000	0.001	0.004	0.93	0.67
	LE	APP	SORL1	0.000	0.001	0.000	0.98	0.86
	LE	APP	TNFRSF21	0.000	0.001	0.000	0.98	0.92
	LE	BMP4	ACVR2A_BMPR1A	0.365	0.051	0.439	0.22	0.65
	LE	BMP4	ACVR2A_BMPR1B	0.365	0.051	0.439	0.22	0.65
	LE	BMP4	ACVR2B_BMPR1A	0.000	0.002	0.706	0.22	0.41
	LE	BMP4	ACVR2B_BMPR1B	0.000	0.002	0.706	0.22	0.41
	LE	BMP4	BMPR1A_BMPR2	0.157	0.036	0.127	0.22	0.86
	LE	BMP4	BMPR1B_BMPR2	0.000	0.001	0.514	0.22	0.37
	LE	CXCL12	DPP4	0.000	0.001	0.018	0.60	0.71

source	target	ligand_complex	receptor_complex	cellphone_pvals	specificity_rank	magnitude_rank	ligand_%_exp	receptor_%_exp
	LE	DKK2	LRP6	0.000	0.001	0.023	0.59	0.86
	LE	FTH1	SCARA5	1.000	1.000	0.025	0.94	0.20
	LE	FTL	SCARA5	1.000	1.000	0.039	0.90	0.20
	LE	GAS6	AXL	1.000	1.000	0.475	0.43	0.29
	LE	GAS6	TYRO3	1.000	0.418	0.475	0.43	0.47
	LE	GPI	AMFR	0.006	0.017	0.046	0.78	0.71
	LE	HBEGF	EGFR	0.977	0.973	0.130	0.32	0.82
	LE	HBEGF	ERBB2	0.000	0.001	0.371	0.32	0.71
	LE	HBEGF	ERBB4	0.000	0.000	0.048	0.32	0.51
	LE	HDGF	NCL	0.002	0.014	0.001	0.71	0.96
	LE	HFE	TFRC	0.979	0.488	0.304	0.43	0.57
	LE	HSD17B12	AR	1.000	1.000	0.404	0.53	0.29
	LE	IGF1	GPC3_IGF1R	1.000	1.000	0.437	0.45	0.24
	LE	IGF1	IGF1R	1.000	1.000	0.004	0.45	0.98
	LE	IGF1	IGFBP3	0.154	0.362	0.001	0.45	0.90
	LE	IGF1	IGFBP4	1.000	1.000	0.367	0.45	0.47
	LE	IGF1	IGFBP5	1.000	1.000	0.372	0.45	0.41
	LE	IGF1	IGFBP6	1.000	1.000	0.392	0.45	0.37
	LE	IGF1	ITGA6_ITGB4	1.000	1.000	0.040	0.45	0.88
	LE	IGFBP3	TMEM219	1.000	1.000	0.218	0.48	0.51
	LE	KITLG	KIT	0.000	0.001	0.005	0.91	0.41
	LE	LIPA	RORA	1.000	1.000	0.040	0.59	0.86
	LE	LIPA	RORC	0.000	0.001	0.340	0.59	0.45
	LE	PDGFB	PDGFRA	0.973	0.933	0.122	0.75	0.22
	LE	PDGFC	PDGFRA	1.000	1.000	0.053	0.73	0.22
	LE	PPIA	BSG	0.000	0.005	0.000	0.94	0.98
	LE	PTGES3	PTGER2	0.000	0.001	0.011	0.77	0.80
	LE	PTGES3	PTGER4	0.579	0.273	0.046	0.77	0.67
	LE	SRD5A3	AR	1.000	1.000	0.794	0.29	0.29
	LE	TGFA	EGFR	0.980	0.719	0.152	0.32	0.82
	LE	TGFB1	ACVR1_TGFBR2	0.000	0.001	0.094	0.65	0.82
	LE	TGFB1	ITGAV_ITGB6	0.000	0.003	0.026	0.65	0.27
	LE	TGFB1	TGFBR1_TGFBR2	0.000	0.005	0.103	0.65	0.78
	LE	TGFB1	TGFBR3	1.000	1.000	0.103	0.65	0.76
	LE	TGFB2	ACVR1_TGFBR2	0.687	0.284	0.157	0.44	0.82
	LE	TGFB2	TGFBR1_TGFBR2	0.992	1.000	0.175	0.44	0.78
	LE	TGFB2	TGFBR3	1.000	1.000	0.175	0.44	0.76
	LE	TGM2	ADGRG1	0.000	0.001	0.024	0.77	0.69
	LE	TNFSF10	TNFRSF10A	0.000	0.001	0.131	0.70	0.45
	LE	TNFSF12	TNFRSF12A	0.744	0.330	0.568	0.46	0.33
	LE	UBASH3B	ESR1	0.309	0.430	0.002	0.44	1.00
	MPH	AGRN	PTPRS	0.974	0.132	0.291	0.47	0.35
	MPH	APLP2	PLXNA4	0.000	0.002	0.004	0.93	0.33
	MPH	APOE	TREM2_TYROBP	0.000	0.001	0.006	0.80	0.43
	MPH	APP	PLXNA4	0.000	0.002	0.001	0.98	0.33
	MPH	APP	SORL1	0.000	0.001	0.001	0.98	0.44
	MPH	APP	TNFRSF21	0.000	0.001	0.001	0.98	0.41
	MPH	APP	TREM2_TYROBP	0.000	0.000	0.001	0.98	0.43
	MPH	CLU	TREM2_TYROBP	0.000	0.000	0.005	0.82	0.43
	MPH	CSF1	CSF1R	0.000	0.000	0.020	0.27	0.73
	MPH	CXCL12	CXCR4	0.000	0.000	0.021	0.60	0.43
	MPH	DKK2	LRP6	0.000	0.001	0.023	0.59	0.46
	MPH	GAS6	AXL	1.000	1.000	0.148	0.43	0.45
	MPH	GAS6	MERTK	1.000	1.000	0.145	0.43	0.38
	MPH	GPI	AMFR	0.000	0.005	0.044	0.78	0.41
	MPH	HDGF	NCL	1.000	1.000	0.006	0.71	0.78

source	target	ligand_complex	receptor_complex	cellphone_pvals	specificity_rank	magnitude_rank	ligand_%_exp	receptor_%_exp
	MPH	IGF1	IGF1R	1.000	1.000	0.130	0.45	0.43
	MPH	IGF1	IGFBP4	1.000	1.000	0.094	0.45	0.48
	MPH	IGFBP3	TMEM219	0.961	0.183	0.157	0.48	0.31
	MPH	LIPA	RORA	1.000	1.000	0.248	0.59	0.24
	MPH	PPIA	BSG	1.000	1.000	0.000	0.94	0.72
	MPH	PTGES3	PTGER4	0.000	0.002	0.022	0.77	0.47
	MPH	RNASET2	TLR8	0.000	0.001	0.410	0.34	0.34
	MPH	SEMA3F	NRP2	0.000	0.001	0.178	0.54	0.31
	MPH	TGFB1	ACVR1_TGFBR2	0.000	0.007	0.161	0.65	0.30
	MPH	TGFB1	TGFBR1_TGFBR2	0.000	0.001	0.028	0.65	0.56
	MPH	TGFB2	ACVR1_TGFBR2	1.000	1.000	0.294	0.44	0.30
	MPH	TGFB2	TGFBR1_TGFBR2	0.000	0.003	0.050	0.44	0.56
	MPH	UBASH3B	ESR1	1.000	1.000	0.306	0.44	0.27
	MPH	VEGFB	NRP1	0.000	0.050	0.038	0.22	0.67

Table S2.1. Significant L-R interactions between major cell categories in *Mus musculus* 4.5dpc.

Secreted signaling and small molecule mediated.

(LE= luminal epithelium, ESF= endometrial stromal fibroblast, MPH= macrophage, VEC= vascular endothelial cell)

source	target	ligand_complex	receptor_complex	cellphone_pvals	specificity_rank	magnitude_rank	ligand_%_exp	receptor_%_exp
ESF	LE	ALDH1A1	RORB	0.906	0.771	0.012	0.66	0.48
	LE	ALDH1A2	RORB	0.000	0.010	0.002	0.83	0.48
	LE	APP	SORL1	0.014	0.248	0.005	0.94	0.20
	LE	FGF1	FGFR1	0.965	0.164	0.337	0.27	0.27
	LE	FGF1	FGFR2	0.000	0.000	0.106	0.27	0.44
	LE	FGF2	FGFR1	1.000	1.000	0.185	0.43	0.27
	LE	FGF2	FGFR2	0.001	0.004	0.060	0.43	0.44
	LE	FGF7	FGFR2	0.000	0.000	0.018	0.54	0.44
	LE	GAS6	AXL	0.014	0.007	0.070	0.58	0.31
	LE	GLS_SLC1A3	GRIK1	0.000	0.000	0.039	0.41	0.21
	LE	GLS_SLC1A3	GRM7	1.000	1.000	0.034	0.41	0.33
	LE	GPI	AMFR	0.123	0.024	0.044	0.71	0.27
	LE	HDGF	NCL	0.996	1.000	0.009	0.44	0.80
	LE	HGF	MET	0.921	0.765	0.427	0.30	0.21
	LE	HSD17B1	ESR1	1.000	1.000	0.008	0.62	0.73
	LE	IGF1	GPC3_IGF1R	0.000	0.000	0.001	0.92	0.30
	LE	IGF1	IGF1R	0.000	0.001	0.000	0.92	0.36
	LE	IGF1	ITGA6_ITGB4	0.000	0.001	0.000	0.92	0.39
	LE	IGF2	IGF1R	0.894	0.163	0.079	0.34	0.36
	LE	IGFBP3	TMEM219	0.000	0.000	0.001	0.95	0.23
	LE	PDGFC	PDGFRA	0.005	0.005	0.081	0.48	0.27
	LE	PGF	NRP1	1.000	1.000	0.187	0.21	0.42
	LE	PPIA	BSG	0.000	0.009	0.000	0.93	0.93
	LE	RSPO3	LGR5	1.000	1.000	0.167	0.21	0.27
	LE	TF	TFRC	0.000	0.000	0.156	0.34	0.35
	LE	TGFB1	ACVR1_TGFBR2	1.000	1.000	0.289	0.29	0.36
	LE	TGFB1	TGFBR3	1.000	1.000	0.062	0.29	0.51
	LE	VEGFA	NRP1	1.000	1.000	0.096	0.39	0.42
	LE	VEGFB	NRP1	1.000	1.000	0.047	0.53	0.42
	MPH	ALDH1A1	RORB	1.000	1.000	0.021	0.66	0.37
	MPH	ALDH1A2	RORB	0.000	0.020	0.005	0.83	0.37
	MPH	APOE	TREM2_TYROBI	0.000	0.000	0.000	0.91	0.44
	MPH	APP	SORL1	0.000	0.000	0.000	0.94	0.54
	MPH	APP	TREM2_TYROBI	0.000	0.000	0.001	0.94	0.44
	MPH	CLU	TREM2_TYROBI	0.000	0.000	0.072	0.37	0.44
	MPH	FGF1	FGFR1	0.006	0.006	0.181	0.27	0.41
	MPH	FGF2	FGFR1	0.640	0.057	0.084	0.43	0.41
	MPH	GAS6	AXL	0.493	0.041	0.099	0.58	0.31
	MPH	GDF11	ACVR2A_TGFBI	0.030	0.009	0.158	0.55	0.29
	MPH	GPI	AMFR	0.000	0.006	0.035	0.71	0.44
	MPH	HDGF	NCL	0.991	1.000	0.008	0.44	0.82
	MPH	HSD17B1	ESR1	1.000	1.000	0.024	0.62	0.44
	MPH	HSD17B12	AR	1.000	1.000	0.045	0.61	0.35
	MPH	IGF1	IGF1R	0.000	0.000	0.000	0.92	0.51
	MPH	IGF2	IGF1R	0.040	0.010	0.046	0.34	0.51
	MPH	PGF	NRP1	0.148	0.049	0.023	0.21	0.57
	MPH	PGF	NRP2	0.758	0.733	0.436	0.21	0.28
	MPH	PPIA	BSG	1.000	1.000	0.000	0.93	0.82
	MPH	PTGES3	PTGER2	0.000	0.001	0.005	0.85	0.40
	MPH	PTGES3	PTGER4	0.003	0.005	0.008	0.85	0.21
	MPH	RARRES2	CMKLR1	0.000	0.000	0.097	0.54	0.29
	MPH	SEMA3C	NRP2	0.000	0.105	0.067	0.53	0.28
	MPH	SULT1A1	PPARD	0.000	0.000	0.066	0.30	0.55
	MPH	TF	TFRC	0.000	0.000	0.152	0.34	0.46
	MPH	TGFB1	ACVR1_TGFBR2	1.000	1.000	0.413	0.29	0.31
	MPH	TGFB1	TGFBR1_TGFBR	0.000	0.024	0.208	0.29	0.47
	MPH	TGFB1	TGFBR3	1.000	1.000	0.332	0.29	0.28
	MPH	VEGFA	NRP1	0.001	0.004	0.017	0.39	0.57
	MPH	VEGFA	NRP2	0.002	0.006	0.316	0.39	0.28
	MPH	VEGFB	NRP1	0.664	0.230	0.010	0.53	0.57
LE	ESF	ALDH1A1	CRABP2_RARA	0.000	0.000	0.013	0.78	0.21
	ESF	ALDH1A1	CRABP2_RARA_	0.000	0.000	0.013	0.78	0.21

	ESF	ALDH1A1	CRABP2_RARB	0.000	0.000	0.013	0.78	0.26
	ESF	ALDH1A1	CRABP2_RXRA	0.000	0.000	0.013	0.78	0.26
	ESF	ALDH1A1	RORB	0.000	0.000	0.000	0.78	0.92
	ESF	ALDH1A2	CRABP2_RARA	0.000	0.001	0.199	0.32	0.21
	ESF	ALDH1A2	CRABP2_RARA	0.000	0.001	0.199	0.32	0.21
	ESF	ALDH1A2	CRABP2_RARB	0.000	0.001	0.199	0.32	0.26
	ESF	ALDH1A2	CRABP2_RXRA	0.000	0.001	0.199	0.32	0.26
	ESF	ALDH1A2	RORB	0.000	0.000	0.000	0.32	0.92
	ESF	APP	SORL1	0.999	0.527	0.005	0.81	0.43
	ESF	CXCL12	DPP4	0.000	0.002	0.037	0.54	0.37
	ESF	DHCR24	RORA	0.000	0.003	0.072	0.32	0.51
	ESF	FTH1	SCARA5	0.000	0.000	0.000	0.99	0.33
	ESF	FTL	SCARA5	0.000	0.000	0.000	0.99	0.33
	ESF	GGT1	CYSLTR1	0.000	0.000	0.320	0.36	0.21
	ESF	GPI	AMFR	0.000	0.002	0.022	0.55	0.55
	ESF	HDGF	NCL	0.011	0.008	0.005	0.42	0.85
	ESF	HSD17B1	ESR1	0.000	0.002	0.000	0.48	0.98
	ESF	HSD17B12	AR	0.000	0.001	0.008	0.40	0.73
	ESF	IGF1	GPC3_IGF1R	0.881	0.864	0.021	0.52	0.43
	ESF	IGF1	IGF1R	1.000	1.000	0.022	0.52	0.43
	ESF	IHH	CDON_PTCH1	0.000	0.000	0.071	0.41	0.39
	ESF	LCN2	SLC22A17	0.000	0.000	0.046	0.24	0.73
	ESF	LTB	LTBR	0.112	0.421	0.322	0.30	0.31
	ESF	PGF	NRP1	0.000	0.000	0.010	0.37	0.79
	ESF	PPIA	BSG	0.000	0.010	0.000	0.97	0.84
	ESF	RSPO3	LGR5	0.000	0.000	0.006	0.53	0.40
	ESF	TGFB2	ACVR1_TGFBR2	0.987	1.000	0.060	0.37	0.58
	ESF	TGFB2	TGFBR1_TGFBR	0.733	0.557	0.101	0.37	0.44
	ESF	TGFB2	TGFBR3	0.997	1.000	0.003	0.37	0.88
	ESF	VEGFA	NRP1	0.000	0.001	0.011	0.33	0.79
	ESF	VEGFB	NRP1	0.000	0.001	0.002	0.69	0.79
	TF	ALDH1A1	RORB	0.998	1.000	0.014	0.78	0.22
	TF	ALDH1A2	RORB	1.000	1.000	0.244	0.32	0.22
	TF	APP	SORL1	1.000	1.000	0.006	0.81	0.33
	TF	DHCR24	RORA	0.000	0.001	0.040	0.32	0.56
	TF	GPI	AMFR	0.001	0.005	0.034	0.55	0.41
	TF	HDGF	NCL	0.848	0.095	0.007	0.42	0.79
	TF	HSD17B1	ESR1	0.000	0.001	0.000	0.48	0.99
	TF	HSD17B12	AR	0.000	0.001	0.008	0.40	0.67
	TF	IGF1	GPC3_IGF1R	0.000	0.000	0.000	0.52	0.54
	TF	IGF1	IGF1R	0.827	0.669	0.011	0.52	0.54
	TF	IHH	BOC_PTCH1	0.000	0.000	0.129	0.41	0.21
	TF	LCN2	SLC22A17	0.000	0.000	0.038	0.24	0.69
	TF	LTB	LTBR	0.000	0.016	0.202	0.30	0.34
	TF	PGF	NRP1	0.000	0.001	0.015	0.37	0.65
	TF	PPIA	BSG	0.000	0.007	0.000	0.97	0.87
	TF	TGFB2	ACVR1_TGFBR2	0.999	1.000	0.068	0.37	0.46
	TF	TGFB2	TGFBR1_TGFBR	1.000	1.000	0.225	0.37	0.23
	TF	TGFB2	TGFBR3	0.489	0.490	0.002	0.37	0.84
	TF	VEGFA	NRP1	0.000	0.001	0.017	0.33	0.65
	TF	VEGFB	NRP1	0.000	0.001	0.003	0.69	0.65
	VEC	CCL2	ACKR1	0.000	0.000	0.247	0.22	0.30
	VEC	DHCR24	RORA	1.000	1.000	0.317	0.32	0.23
	VEC	GPI	AMFR	0.003	0.005	0.035	0.55	0.38
	VEC	HDGF	NCL	0.900	0.112	0.007	0.42	0.74
	VEC	HSD17B1	ESR1	1.000	1.000	0.104	0.48	0.20
	VEC	IGF1	GPC3_IGF1R	0.572	0.440	0.018	0.52	0.34
	VEC	IGF1	IGF1R	1.000	1.000	0.034	0.52	0.34
	VEC	IGF1	ITGA6_ITGB4	0.000	0.001	0.001	0.52	0.45
	VEC	IGFBP3	TMEM219	0.000	0.023	0.006	0.83	0.23
	VEC	KITLG	KIT	0.907	0.438	0.347	0.26	0.21
	VEC	LCN2	SLC22A17	0.001	0.004	0.132	0.24	0.46
	VEC	LTB	LTBR	0.000	0.018	0.217	0.30	0.34

	VEC	PGF	FLT1	0.000	0.000	0.001	0.37	0.83
	VEC	PGF	FLT1_KDR	0.000	0.000	0.001	0.37	0.79
	VEC	PGF	NRP1	0.000	0.000	0.001	0.37	0.82
	VEC	PGF	NRP2	0.000	0.001	0.196	0.37	0.27
	VEC	PPIA	BSG	0.000	0.010	0.000	0.97	0.79
	VEC	SEMA3C	NRP2	0.000	0.004	0.044	0.48	0.27
	VEC	TGFB2	ACVR1_TGFBR2	0.998	1.000	0.065	0.37	0.46
	VEC	TGFB2	TGFBR3	1.000	1.000	0.004	0.37	0.73
	VEC	VEGFA	FLT1	0.000	0.000	0.001	0.33	0.83
	VEC	VEGFA	FLT1_KDR	0.000	0.000	0.001	0.33	0.79
	VEC	VEGFA	KDR	0.000	0.000	0.003	0.33	0.79
	VEC	VEGFA	NRP1	0.000	0.000	0.002	0.33	0.82
	VEC	VEGFA	NRP2	0.000	0.001	0.254	0.33	0.27
	VEC	VEGFB	FLT1	0.000	0.000	0.000	0.69	0.83
	VEC	VEGFB	FLT1_KDR	0.000	0.000	0.000	0.69	0.79
	VEC	VEGFB	NRP1	0.000	0.000	0.000	0.69	0.82
MPH	**ESF**	ALDH1A1	CRABP2_RARA	0.990	0.424	0.310	0.30	0.21
	ESF	ALDH1A1	CRABP2_RARA	0.990	0.424	0.310	0.30	0.21
	ESF	ALDH1A1	CRABP2_RARB	0.990	0.424	0.310	0.30	0.26
	ESF	ALDH1A1	CRABP2_RXRA	0.990	0.424	0.310	0.30	0.26
	ESF	ALDH1A1	RORB	0.000	0.002	0.000	0.30	0.92
	ESF	ALDH1A2	CRABP2_RARA	0.000	0.000	0.078	0.35	0.21
	ESF	ALDH1A2	CRABP2_RARA	0.000	0.000	0.078	0.35	0.21
	ESF	ALDH1A2	CRABP2_RARB	0.000	0.000	0.078	0.35	0.26
	ESF	ALDH1A2	CRABP2_RXRA	0.000	0.000	0.078	0.35	0.26
	ESF	ALDH1A2	RORB	0.000	0.000	0.000	0.35	0.92
	ESF	APP	SORL1	1.000	1.000	0.023	0.59	0.43
	ESF	CXCL12	DPP4	1.000	1.000	0.309	0.20	0.37
	ESF	DHCR24	RORA	0.000	0.008	0.110	0.32	0.51
	ESF	FTH1	SCARA5	0.000	0.000	0.000	0.99	0.33
	ESF	FTL	SCARA5	0.000	0.000	0.000	0.99	0.33
	ESF	GAS6	AXL	0.015	0.009	0.074	0.37	0.62
	ESF	GPI	AMFR	0.760	0.074	0.054	0.49	0.55
	ESF	HBEGF	EGFR	0.000	0.000	0.044	0.32	0.59
	ESF	HBEGF	ERBB2	0.000	0.000	0.260	0.32	0.29
	ESF	HDGF	NCL	0.893	0.646	0.007	0.42	0.85
	ESF	HSD17B1	ESR1	0.000	0.003	0.000	0.53	0.98
	ESF	HSD17B12	AR	0.000	0.001	0.007	0.57	0.73
	ESF	HSD17B3	AR	0.000	0.000	0.027	0.20	0.73
	ESF	IGF1	GPC3_IGF1R	0.999	1.000	0.029	0.43	0.43
	ESF	IGF1	IGF1R	1.000	1.000	0.034	0.43	0.43
	ESF	LCN2	SLC22A17	0.000	0.000	0.042	0.20	0.73
	ESF	LIPA	RORA	0.000	0.000	0.010	0.66	0.51
	ESF	LTB	LTBR	0.000	0.023	0.197	0.46	0.31
	ESF	PPIA	BSG	1.000	1.000	0.000	0.96	0.84
	ESF	RSPO3	LGR5	0.536	0.045	0.027	0.21	0.40
	ESF	TGFB1	ACVR1_TGFBR2	0.000	0.000	0.015	0.68	0.58
	ESF	TGFB1	TGFBR1_TGFBR	0.000	0.000	0.021	0.68	0.44
	ESF	TGFB1	TGFBR3	0.000	0.000	0.001	0.68	0.88
	ESF	TNF	TNFRSF1A	0.000	0.002	0.402	0.20	0.41
	ESF	UBASH3B	ESR1	0.000	0.000	0.001	0.30	0.98
	ESF	VEGFB	NRP1	0.000	0.003	0.005	0.58	0.79
	TF	ALDH1A1	RORB	1.000	1.000	0.338	0.30	0.22
	TF	ALDH1A2	RORB	1.000	1.000	0.093	0.35	0.22
	TF	APP	SORL1	1.000	1.000	0.028	0.59	0.33
	TF	DHCR24	RORA	0.000	0.003	0.054	0.32	0.56
	TF	GAS6	AXL	0.000	0.000	0.024	0.37	0.69
	TF	GPI	AMFR	0.999	1.000	0.081	0.49	0.41
	TF	HBEGF	EGFR	0.000	0.000	0.102	0.32	0.36
	TF	HBEGF	ERBB2	0.000	0.000	0.247	0.32	0.23
	TF	HDGF	NCL	1.000	1.000	0.010	0.42	0.79
	TF	HSD17B1	ESR1	0.000	0.001	0.000	0.53	0.99
	TF	HSD17B12	AR	0.000	0.001	0.007	0.57	0.67

	TF	HSD17B3	AR	0.000	0.000	0.029	0.20	0.67
	TF	IGF1	GPC3_IGF1R	0.000	0.000	0.000	0.43	0.54
	TF	IGF1	IGF1R	0.999	1.000	0.015	0.43	0.54
	TF	LCN2	SLC22A17	0.000	0.000	0.035	0.20	0.69
	TF	LIPA	RORA	0.000	0.000	0.006	0.66	0.56
	TF	LTB	LTBR	0.000	0.007	0.113	0.46	0.34
	TF	PPIA	BSG	0.607	0.201	0.000	0.96	0.87
	TF	TGFB1	ACVR1_TGFBR2	0.000	0.000	0.017	0.68	0.46
	TF	TGFB1	TGFBR1_TGFBR	0.000	0.003	0.038	0.68	0.23
	TF	TGFB1	TGFBR3	0.000	0.000	0.000	0.68	0.84
	TF	TNF	TNFRSF1A	0.000	0.000	0.262	0.20	0.48
	TF	UBASH3B	ESR1	0.000	0.000	0.001	0.30	0.99
	TF	VEGFB	NRP1	0.053	0.012	0.007	0.58	0.65
	VEC	ANGPT1	TEK	0.000	0.000	0.029	0.28	0.62
	VEC	CCL2	ACKR1	0.000	0.000	0.091	0.25	0.30
	VEC	DHCR24	RORA	1.000	1.000	0.404	0.32	0.23
	VEC	GPI	AMFR	1.000	1.000	0.087	0.49	0.38
	VEC	HDGF	NCL	1.000	1.000	0.010	0.42	0.74
	VEC	HSD17B1	ESR1	1.000	1.000	0.150	0.53	0.20
	VEC	IGF1	GPC3_IGF1R	0.975	1.000	0.024	0.43	0.34
	VEC	IGF1	IGF1R	1.000	1.000	0.046	0.43	0.34
	VEC	IGF1	ITGA6_ITGB4	0.000	0.002	0.001	0.43	0.45
	VEC	IGFBP3	TMEM219	1.000	1.000	0.071	0.45	0.23
	VEC	LCN2	SLC22A17	0.000	0.001	0.117	0.20	0.46
	VEC	LIPA	RORA	0.000	0.001	0.025	0.66	0.23
	VEC	LTB	LTBR	0.000	0.008	0.119	0.46	0.34
	VEC	PPIA	BSG	0.999	0.987	0.000	0.96	0.79
	VEC	TBXAS1	TBXA2R	0.000	0.000	0.008	0.72	0.20
	VEC	TGFB1	ACVR1_TGFBR2	0.000	0.000	0.016	0.68	0.46
	VEC	TGFB1	TGFBR3	0.000	0.000	0.001	0.68	0.73
	VEC	TNF	TNFRSF1A	0.000	0.000	0.313	0.20	0.41
	VEC	UBASH3B	ESR1	1.000	1.000	0.365	0.30	0.20
	VEC	VEGFB	FLT1	0.000	0.000	0.000	0.58	0.83
	VEC	VEGFB	FLT1_KDR	0.000	0.000	0.000	0.58	0.79
	VEC	VEGFB	NRP1	0.000	0.001	0.001	0.58	0.82
	VEC	VEGFC	FLT4	0.198	0.038	0.456	0.21	0.22
	VEC	VEGFC	KDR	0.000	0.000	0.005	0.21	0.79
TF	**LE**	ADM	RAMP3	0.000	0.000	0.021	0.30	0.56
	LE	ALDH1A1	RORB	1.000	1.000	0.021	0.49	0.48
	LE	ALDH1A2	RORB	1.000	1.000	0.050	0.22	0.48
	LE	APP	SORL1	1.000	1.000	0.006	0.90	0.20
	LE	BMPR1B_BMPR2	BMPR1A	0.000	0.000	0.338	0.22	0.27
	LE	CXCL12	DPP4	0.000	0.000	0.020	0.55	0.35
	LE	FGF1	FGFR1	0.630	0.056	0.291	0.24	0.27
	LE	FGF1	FGFR2	0.000	0.000	0.088	0.24	0.44
	LE	FGF2	FGFR1	0.000	0.001	0.023	0.65	0.27
	LE	FGF2	FGFR2	0.000	0.000	0.011	0.65	0.44
	LE	GAS6	AXL	0.970	0.428	0.137	0.38	0.31
	LE	GLS_SLC1A3	GRIK1	0.000	0.000	0.037	0.44	0.21
	LE	GLS_SLC1A3	GRM7	1.000	1.000	0.029	0.44	0.33
	LE	GPI	AMFR	1.000	1.000	0.089	0.54	0.27
	LE	HDGF	NCL	0.980	0.800	0.008	0.38	0.80
	LE	HGF	MET	0.998	1.000	0.485	0.20	0.21
	LE	HSD17B1	ESR1	1.000	1.000	0.007	0.51	0.73
	LE	IGF1	GPC3_IGF1R	0.010	0.145	0.012	0.72	0.30
	LE	IGF1	IGF1R	0.011	0.011	0.006	0.72	0.36
	LE	IGF1	ITGA6_ITGB4	0.000	0.108	0.006	0.72	0.39
	LE	IGF2	IGF1R	0.000	0.000	0.016	0.57	0.36
	LE	IGFBP3	TMEM219	0.000	0.000	0.002	0.89	0.23
	LE	PPIA	BSG	0.001	0.009	0.000	0.95	0.93
	LE	RSPO3	LGR5	0.000	0.003	0.007	0.70	0.27
	LE	TF	TFRC	0.000	0.000	0.197	0.23	0.35
	LE	TGFB1	ACVR1_TGFBR2	0.999	1.000	0.259	0.25	0.36

	LE	TGFB1	TGFBR3	1.000	1.000	0.052	0.25	0.51
	LE	TGFB2	ACVR1_TGFBR2	1.000	1.000	0.063	0.41	0.36
	LE	TGFB2	TGFBR3	1.000	1.000	0.019	0.41	0.51
	LE	VEGFA	NRP1	1.000	1.000	0.079	0.31	0.42
	LE	VEGFB	NRP1	0.913	0.785	0.011	0.70	0.42
	MPH	ADM	RAMP3	0.100	0.015	0.198	0.30	0.21
	MPH	ALDH1A1	RORB	1.000	1.000	0.042	0.49	0.37
	MPH	ALDH1A2	RORB	1.000	1.000	0.118	0.22	0.37
	MPH	APOE	TREM2_TYROBP	0.000	0.000	0.000	0.99	0.44
	MPH	APP	SORL1	0.000	0.000	0.001	0.90	0.54
	MPH	APP	TREM2_TYROBP	0.000	0.000	0.001	0.90	0.44
	MPH	CLU	TREM2_TYROBP	0.000	0.000	0.011	0.68	0.44
	MPH	CSF1	CSF1R	0.000	0.000	0.317	0.22	0.37
	MPH	CXCL12	DPP4	0.000	0.001	0.026	0.55	0.33
	MPH	FGF1	FGFR1	0.000	0.001	0.129	0.24	0.41
	MPH	FGF2	FGFR1	0.000	0.000	0.015	0.65	0.41
	MPH	GAS6	AXL	1.000	1.000	0.218	0.38	0.31
	MPH	GDF11	ACVR2A_TGFBR	1.000	1.000	0.426	0.23	0.29
	MPH	GPI	AMFR	0.960	0.635	0.064	0.54	0.44
	MPH	HDGF	NCL	0.951	0.768	0.007	0.38	0.82
	MPH	HSD17B1	ESR1	1.000	1.000	0.024	0.51	0.44
	MPH	HSD17B12	AR	1.000	1.000	0.044	0.55	0.35
	MPH	IGF1	IGF1R	0.000	0.003	0.004	0.72	0.51
	MPH	IGF2	IGF1R	0.000	0.000	0.011	0.57	0.51
	MPH	PPIA	BSG	1.000	1.000	0.000	0.95	0.82
	MPH	PTGES3	PTGER2	0.000	0.001	0.005	0.87	0.40
	MPH	PTGES3	PTGER4	0.000	0.001	0.008	0.87	0.21
	MPH	SEMA3C	NRP2	0.000	0.000	0.003	0.76	0.28
	MPH	TF	TFRC	0.000	0.000	0.193	0.23	0.46
	MPH	TGFB1	ACVR1_TGFBR2	1.000	1.000	0.386	0.25	0.31
	MPH	TGFB1	TGFBR1_TGFBR	0.000	0.017	0.189	0.25	0.47
	MPH	TGFB1	TGFBR3	1.000	1.000	0.310	0.25	0.28
	MPH	TGFB2	ACVR1_TGFBR2	1.000	1.000	0.109	0.41	0.31
	MPH	TGFB2	TGFBR1_TGFBR	0.000	0.006	0.047	0.41	0.47
	MPH	TGFB2	TGFBR3	1.000	1.000	0.073	0.41	0.28
	MPH	VEGFA	NRP1	0.000	0.001	0.015	0.31	0.57
	MPH	VEGFA	NRP2	0.000	0.002	0.288	0.31	0.28
	MPH	VEGFB	NRP1	0.000	0.001	0.002	0.70	0.57
VEC	**LE**	APP	SORL1	0.000	0.010	0.003	0.91	0.20
	LE	CXCL12	DPP4	0.271	0.025	0.108	0.20	0.35
	LE	FGF2	FGFR1	1.000	1.000	0.191	0.35	0.27
	LE	FGF2	FGFR2	0.001	0.004	0.062	0.35	0.44
	LE	GAS6	AXL	0.000	0.002	0.054	0.50	0.31
	LE	GLS_SLC1A1	GRIK1	0.000	0.000	0.018	0.26	0.21
	LE	GLS_SLC1A1	GRM7	1.000	1.000	0.016	0.26	0.33
	LE	GPI	AMFR	1.000	1.000	0.227	0.37	0.27
	LE	HBEGF	ERBB2	0.000	0.000	0.353	0.22	0.27
	LE	HDGF	NCL	0.991	0.790	0.008	0.37	0.80
	LE	HSD17B1	ESR1	1.000	1.000	0.012	0.38	0.73
	LE	IGFBP3	TMEM219	1.000	1.000	0.184	0.35	0.23
	LE	LTB	LTBR	0.000	0.001	0.023	0.57	0.34
	LE	PDGFB	PDGFRA	0.000	0.001	0.297	0.32	0.27
	LE	PPIA	BSG	0.000	0.007	0.000	0.94	0.93
	LE	TGFB1	ACVR1_TGFBR2	0.132	0.017	0.112	0.38	0.36
	LE	TGFB1	TGFBR3	1.000	1.000	0.029	0.38	0.51
	LE	UBASH3B	ESR1	1.000	0.732	0.014	0.32	0.73
	LE	VEGFB	NRP1	1.000	1.000	0.137	0.22	0.42
	MPH	APOE	TREM2_TYROBP	1.000	0.021	0.002	0.72	0.44
	MPH	APP	SORL1	0.000	0.000	0.000	0.91	0.54
	MPH	APP	TREM2_TYROBP	0.000	0.000	0.000	0.91	0.44
	MPH	CLU	TREM2_TYROBP	0.000	0.000	0.009	0.61	0.44
	MPH	CXCL12	DPP4	0.995	0.248	0.186	0.20	0.33
	MPH	FGF2	FGFR1	0.702	0.076	0.089	0.35	0.41

	MPH	GAS6	AXL	0.039	0.010	0.075	0.50	0.31
	MPH	GPI	AMFR	1.000	1.000	0.149	0.37	0.44
	MPH	HDGF	NCL	0.981	0.639	0.008	0.37	0.82
	MPH	HSD17B1	ESR1	1.000	1.000	0.044	0.38	0.44
	MPH	HSD17B12	AR	1.000	1.000	0.062	0.49	0.35
	MPH	HSD17B3	AR	1.000	0.678	0.300	0.22	0.35
	MPH	LTB	LTBR	0.000	0.001	0.030	0.57	0.33
	MPH	PPIA	BSG	1.000	1.000	0.000	0.94	0.82
	MPH	PTGES3	PTGER2	0.000	0.000	0.002	0.82	0.40
	MPH	PTGES3	PTGER4	0.000	0.001	0.005	0.82	0.21
	MPH	SEMA3F	NRP2	0.000	0.000	0.312	0.30	0.28
	MPH	TGFB1	ACVR1_TGFBR2	0.999	0.488	0.237	0.38	0.31
	MPH	TGFB1	TGFBR1_TGFBR	0.000	0.003	0.088	0.38	0.47
	MPH	TGFB1	TGFBR3	1.000	1.000	0.145	0.38	0.28
	MPH	UBASH3B	ESR1	1.000	1.000	0.052	0.32	0.44
	MPH	VEGFB	NRP1	1.000	1.000	0.020	0.22	0.57

Table S2.2. Significant L-R interactions between major cell categories in *Cavia Porcellus* 6.5dpc. Secreted signaling and small molecule mediated.

source	target	ligand_complex	receptor_complex	cellphone_pvals	specificity_rank	magnitude_rank	ligand_%_exp	receptor_%_exp
ESF-SMOC2	LE-OAT	HDGF	NCL	0.00	0.01	0.02	0.24	0.53
	LE-OAT	IGF1	IGFBP2	0.00	0.09	0.00	0.98	0.28
	LE-OAT	IGFBP3	TMEM219	0.00	0.00	0.05	0.29	0.24
	LE-OAT	PPIA	BSG	0.00	0.00	0.00	0.85	0.33
	LE-SLCO2A1	APP	SORL1	0.00	0.00	0.02	0.43	0.52
	LE-SLCO2A1	BMP2	ACVR2A_BMPR1B	0.00	0.00	0.01	0.62	0.40
	LE-SLCO2A1	BMP2	ACVR2B_BMPR1B	0.00	0.00	0.02	0.62	0.30
	LE-SLCO2A1	BMP2	BMPR1B_BMPR2	0.00	0.00	0.01	0.62	0.28
	LE-SLCO2A1	BMP4	ACVR2A_BMPR1B	0.00	0.00	0.08	0.20	0.40
	LE-SLCO2A1	BMP4	ACVR2B_BMPR1B	0.00	0.00	0.14	0.20	0.30
	LE-SLCO2A1	BMP4	BMPR1B_BMPR2	0.21	0.02	0.11	0.20	0.28
	LE-SLCO2A1	GAS6	MERTK	0.00	0.00	0.03	0.40	0.40
	LE-SLCO2A1	GPI	AMFR	0.00	0.00	0.03	0.23	0.62
	LE-SLCO2A1	HDGF	NCL	0.94	1.00	0.03	0.24	0.58
	LE-SLCO2A1	IGF1	IGF1R	0.00	0.02	0.00	0.98	0.49
	LE-SLCO2A1	IGF1	IGFBP2	0.00	0.22	0.00	0.98	0.23
	LE-SLCO2A1	IGF1	ITGA6_ITGB4	0.00	0.00	0.00	0.98	0.34
	LE-SLCO2A1	IGFBP3	TMEM219	0.00	0.00	0.07	0.29	0.23
	LE-SLCO2A1	LGALS3	MERTK	0.00	0.00	0.01	0.54	0.40
	LE-SLCO2A1	PPIA	BSG	0.00	0.00	0.00	0.85	0.76
	LE-SLCO2A1	WNT6	FRZB	0.00	0.00	0.03	0.46	0.37
	MPH	ANXA1	FPR2	0.00	0.00	0.00	0.88	0.72
	MPH	APP	FPR2	0.00	0.00	0.00	0.43	0.72
	MPH	APP	SORL1	0.00	0.00	0.02	0.43	0.44
	MPH	APP	TNFRSF21	0.00	0.00	0.05	0.43	0.23
	MPH	GAS6	MERTK	0.00	0.00	0.01	0.40	0.62
	MPH	GPI	AMFR	0.53	0.30	0.06	0.23	0.39
	MPH	HDGF	NCL	0.00	0.00	0.02	0.24	0.65
	MPH	IGF1	IGF1R	0.00	0.00	0.00	0.98	0.57
	MPH	IGF1	IGFBP2	0.00	0.12	0.00	0.98	0.30
	MPH	IGFBP3	TMEM219	0.00	0.00	0.02	0.29	0.45
	MPH	LGALS3	MERTK	0.00	0.00	0.00	0.54	0.62
	MPH	PGF	NRP1	0.00	0.00	0.01	0.49	0.49
	MPH	PGF	NRP2	0.00	0.00	0.02	0.49	0.39
	MPH	PPIA	BSG	0.00	0.01	0.00	0.85	0.24
	MPH	RARRES2	CCRL2	0.00	0.00	0.00	0.84	0.60
	MPH	RNASET2	TLR8	0.00	0.00	0.04	0.46	0.30
	MPH	SEMA3A	NRP1	0.00	0.00	0.00	0.56	0.49
	MPH	SEMA3A	NRP1_PLXNA1	0.00	0.00	0.00	0.56	0.27
	MPH	SEMA3C	NRP2	0.00	0.00	0.01	0.44	0.39
	MPH	VEGFA	NRP1	0.00	0.00	0.01	0.41	0.49
	MPH	VEGFA	NRP2	0.00	0.00	0.03	0.41	0.39
LE-OAT	ESF-SMOC2	FTH1	SCARA5	0.00	0.00	0.00	1.00	0.83
	ESF-SMOC2	FTL	SCARA5	0.00	0.00	0.00	0.97	0.83
	ESF-SMOC2	IGF1	IGF1R	0.78	1.00	0.00	0.82	0.48
	ESF-SMOC2	IGF1	IGFBP2	0.00	0.00	0.00	0.82	0.91
	ESF-SMOC2	IGF1	IGFBP3	0.00	0.00	0.00	0.82	0.29
	ESF-SMOC2	NTF3	NGFR	1.00	0.50	0.06	0.20	0.33
	ESF-SMOC2	NTF3	NTRK2	0.00	0.01	0.01	0.20	0.72
	ESF-SMOC2	PPIA	BSG	0.00	0.21	0.00	0.73	0.42
	ESF-SMOC2	SRD5A1	AR	0.00	0.00	0.01	0.59	0.46
	VEC	GGT1	CYSLTR1	0.00	0.00	0.13	0.26	0.21
	VEC	IGF1	IGF1R	0.00	0.10	0.00	0.82	0.59
	VEC	IGF1	IGFBP2	0.00	0.00	0.00	0.82	0.92
	VEC	IGF1	IGFBP3	0.00	0.00	0.00	0.82	0.58
	VEC	IGF1	ITGA6_ITGB4	0.00	0.00	0.00	0.82	0.37
	VEC	NTF3	NTRK3	1.00	1.00	0.07	0.20	0.31
	VEC	PPIA	BSG	0.00	0.01	0.00	0.73	0.46
	VEC	SRD5A1	AR	0.00	0.00	0.01	0.59	0.27
LE-SLCO2A1	ESF-SMOC2	APP	SORL1	0.00	0.06	0.03	0.55	0.31
	ESF-SMOC2	EGF	EGFR	0.00	0.00	0.00	0.33	0.91
	ESF-SMOC2	FTH1	SCARA5	0.00	0.00	0.00	1.00	0.83

source	target	ligand_complex	receptor_complex	cellphone_pvals	specificity_rank	magnitude_rank	ligand_%_exp	receptor_%_exp
	ESF-SMOC2	FTL	SCARA5	0.00	0.00	0.00	0.97	0.83
	ESF-SMOC2	GPI	AMFR	0.00	0.00	0.04	0.39	0.50
	ESF-SMOC2	HDGF	NCL	0.85	0.82	0.03	0.24	0.62
	ESF-SMOC2	HSD17B12	AR	0.00	0.00	0.05	0.21	0.46
	ESF-SMOC2	IGF1	IGF1R	1.00	1.00	0.01	0.71	0.48
	ESF-SMOC2	IGF1	IGFBP2	0.00	0.00	0.00	0.71	0.91
	ESF-SMOC2	IGF1	IGFBP3	1.00	1.00	0.01	0.71	0.29
	ESF-SMOC2	LGALS3	MERTK	0.00	0.00	0.02	0.39	0.52
	ESF-SMOC2	LIF	IL6ST_LIFR	0.00	0.00	0.01	0.21	0.32
	ESF-SMOC2	PDGFC	PDGFRA	0.00	0.00	0.00	0.82	0.49
	ESF-SMOC2	PGF	NRP1	0.00	0.00	0.01	0.72	0.33
	ESF-SMOC2	PPIA	BSG	0.42	0.70	0.00	0.82	0.42
	ESF-SMOC2	SRD5A1	AR	0.01	0.01	0.02	0.49	0.46
	ESF-SMOC2	TGFA	EGFR	0.00	0.00	0.00	0.43	0.91
	ESF-SMOC2	VEGFA	NRP1	0.00	0.00	0.00	0.85	0.33
	VEC	EGF	EGFR	1.00	0.06	0.05	0.33	0.36
	VEC	GPI	AMFR	0.00	0.00	0.03	0.39	0.55
	VEC	HDGF	NCL	0.00	0.00	0.01	0.24	0.75
	VEC	HSD17B12	AR	0.01	0.01	0.15	0.21	0.27
	VEC	IGF1	IGF1R	1.00	1.00	0.00	0.71	0.59
	VEC	IGF1	IGFBP2	0.00	0.02	0.00	0.71	0.92
	VEC	IGF1	IGFBP3	0.45	0.16	0.00	0.71	0.58
	VEC	IGF1	ITGA6_ITGB4	1.00	0.69	0.00	0.71	0.37
	VEC	LGALS3	MERTK	0.78	1.00	0.08	0.39	0.24
	VEC	LIF	IL6ST_LIFR	0.00	0.00	0.00	0.21	0.66
	VEC	PGF	FLT1	0.00	0.00	0.00	0.72	1.00
	VEC	PGF	FLT1_KDR	0.00	0.00	0.00	0.72	0.83
	VEC	PGF	NRP1	0.00	0.00	0.00	0.72	0.79
	VEC	PGF	NRP2	0.00	0.00	0.00	0.72	0.48
	VEC	PPIA	BSG	0.24	0.35	0.00	0.82	0.46
	VEC	SRD5A1	AR	1.00	1.00	0.05	0.49	0.27
	VEC	TGFA	EGFR	1.00	1.00	0.04	0.43	0.36
	VEC	VEGFA	FLT1	0.00	0.00	0.00	0.85	1.00
	VEC	VEGFA	FLT1_KDR	0.00	0.00	0.00	0.85	0.83
	VEC	VEGFA	KDR	0.00	0.00	0.00	0.85	0.83
	VEC	VEGFA	NRP1	0.00	0.00	0.00	0.85	0.79
	VEC	VEGFA	NRP2	0.00	0.00	0.00	0.85	0.48
MPH	**ESF-SMOC2**	ALDH1A1	CRABP2_RARB	0.00	0.00	0.02	0.37	0.45
	ESF-SMOC2	ALDH1A1	CRABP2_RXRA	0.00	0.00	0.02	0.37	0.26
	ESF-SMOC2	ALDH1A1	RORB	0.00	0.00	0.00	0.37	0.70
	ESF-SMOC2	CXCL2	DPP4	0.00	0.00	0.01	0.34	0.39
	ESF-SMOC2	FTH1	SCARA5	0.00	0.00	0.00	1.00	0.83
	ESF-SMOC2	FTL	SCARA5	0.00	0.00	0.00	0.92	0.83
	ESF-SMOC2	GAS6	MERTK	0.00	0.00	0.03	0.20	0.52
	ESF-SMOC2	HBEGF	EGFR	0.00	0.00	0.00	0.31	0.91
	ESF-SMOC2	IGF1	IGF1R	1.00	1.00	0.00	0.82	0.48
	ESF-SMOC2	IGF1	IGFBP2	0.00	0.00	0.00	0.82	0.91
	ESF-SMOC2	IGF1	IGFBP3	0.86	0.46	0.00	0.82	0.29
	ESF-SMOC2	LGALS3	MERTK	0.00	0.00	0.00	0.69	0.52
	ESF-SMOC2	LIPA	RORA	0.00	0.00	0.00	0.24	0.96
	ESF-SMOC2	PDGFC	PDGFRA	0.00	0.00	0.00	0.61	0.49
	ESF-SMOC2	PPIA	BSG	0.00	0.08	0.00	0.83	0.42
	ESF-SMOC2	PSAP	GPR37	0.00	0.00	0.04	0.46	0.22
	ESF-SMOC2	RARRES2	CCRL2	0.37	0.26	0.07	0.31	0.21
	ESF-SMOC2	RARRES2	GPR1	0.00	0.02	0.07	0.31	0.26
	ESF-SMOC2	SRD5A1	AR	0.00	0.00	0.01	0.52	0.46
	ESF-SMOC2	TGFB1	ACVR1_TGFBR2	0.00	0.00	0.01	0.59	0.42
	ESF-SMOC2	TGFB1	TGFBR3	0.00	0.00	0.00	0.59	0.81
	ESF-SMOC2	TNF	TNFRSF1A	0.00	0.00	0.02	0.26	0.62
	ESF-SMOC2	UBASH3B	ESR1	0.00	0.00	0.00	0.57	0.96
	VEC	ALDH1A1	RORB	0.01	0.16	0.04	0.37	0.36

source	target	ligand_complex	receptor_complex	cellphone_pvals	specificity_rank	magnitude_rank	ligand_%_exp	receptor_%_exp
	VEC	CXCL2	DPP4	0.01	0.01	0.02	0.34	0.37
	VEC	GAS6	MERTK	0.32	0.23	0.13	0.20	0.24
	VEC	HBEGF	EGFR	1.00	0.63	0.04	0.31	0.36
	VEC	HGF	MET	0.00	0.00	0.02	0.23	0.55
	VEC	IGF1	IGF1R	1.00	1.00	0.00	0.82	0.59
	VEC	IGF1	IGFBP2	0.00	0.00	0.00	0.82	0.92
	VEC	IGF1	IGFBP3	0.00	0.00	0.00	0.82	0.58
	VEC	IGF1	ITGA6_ITGB4	0.01	0.10	0.00	0.82	0.37
	VEC	LGALS3	MERTK	0.00	0.00	0.01	0.69	0.24
	VEC	LIPA	RORA	0.02	0.12	0.04	0.24	0.36
	VEC	LTC4S	CYSLTR1	0.00	0.00	0.12	0.27	0.21
	VEC	PPIA	BSG	0.00	0.00	0.00	0.83	0.46
	VEC	RARRES2	CCRL2	0.00	0.00	0.02	0.31	0.54
	VEC	SRD5A1	AR	0.60	0.32	0.03	0.52	0.27
	VEC	TGFB1	ACVR1_TGFBR2	0.00	0.00	0.02	0.59	0.39
	VEC	TGFB1	TGFBR3	0.00	0.00	0.01	0.59	0.53
	VEC	TNF	TNFRSF1A	0.00	0.00	0.02	0.26	0.64
	VEC	UBASH3B	ESR1	1.00	0.03	0.00	0.57	0.48
VEC	**LE-OAT**	CXCL12	DPP4	0.00	0.00	0.01	0.22	0.62
	LE-OAT	CXCL2	DPP4	0.00	0.00	0.00	0.47	0.62
	LE-OAT	HDGF	NCL	0.00	0.00	0.02	0.36	0.53
	LE-OAT	IGF1	IGFBP2	1.00	1.00	0.01	0.72	0.28
	LE-OAT	IGFBP3	TMEM219	0.00	0.00	0.01	0.58	0.24
	LE-OAT	PPIA	BSG	0.00	0.00	0.00	0.89	0.33
	LE-OAT	UBASH3B	ESR1	1.00	1.00	0.07	0.24	0.30
	LE-SLCO2A1	APP	SORL1	0.00	0.00	0.01	0.61	0.52
	LE-SLCO2A1	CXCL12	DPP4	1.00	0.26	0.13	0.22	0.25
	LE-SLCO2A1	CXCL2	DPP4	1.00	0.39	0.03	0.47	0.25
	LE-SLCO2A1	GAS6	MERTK	0.00	0.00	0.04	0.32	0.40
	LE-SLCO2A1	GPI	AMFR	0.00	0.00	0.03	0.30	0.62
	LE-SLCO2A1	HBEGF	EGFR	0.00	0.00	0.00	0.77	0.91
	LE-SLCO2A1	HBEGF	ERBB2	0.00	0.00	0.01	0.77	0.28
	LE-SLCO2A1	HBEGF	ERBB4	0.00	0.00	0.01	0.77	0.31
	LE-SLCO2A1	HDGF	NCL	0.15	0.03	0.03	0.36	0.58
	LE-SLCO2A1	IGF1	IGF1R	1.00	1.00	0.01	0.72	0.49
	LE-SLCO2A1	IGF1	IGFBP2	1.00	1.00	0.01	0.72	0.23
	LE-SLCO2A1	IGF1	ITGA6_ITGB4	1.00	1.00	0.01	0.72	0.34
	LE-SLCO2A1	IGFBP3	TMEM219	0.00	0.00	0.02	0.58	0.23
	LE-SLCO2A1	LGALS3	MERTK	0.01	0.14	0.05	0.27	0.40
	LE-SLCO2A1	PPIA	BSG	0.00	0.00	0.00	0.89	0.76
	LE-SLCO2A1	TNFSF10	TNFRSF10A	0.81	0.11	0.02	0.47	0.48
	LE-SLCO2A1	UBASH3B	ESR1	1.00	1.00	0.01	0.24	0.72
	MPH	ANXA1	FPR2	0.00	0.00	0.00	0.81	0.72
	MPH	APP	FPR2	0.00	0.00	0.00	0.61	0.72
	MPH	APP	SORL1	0.00	0.00	0.01	0.61	0.44
	MPH	APP	TNFRSF21	0.00	0.00	0.02	0.61	0.23
	MPH	CXCL12	CXCR4	0.00	0.00	0.06	0.22	0.31
	MPH	GAS6	MERTK	0.00	0.00	0.01	0.32	0.62
	MPH	GPI	AMFR	0.00	0.01	0.05	0.30	0.39
	MPH	HDGF	NCL	0.00	0.00	0.01	0.36	0.65
	MPH	IGF1	IGF1R	1.00	1.00	0.00	0.72	0.57
	MPH	IGF1	IGFBP2	1.00	1.00	0.01	0.72	0.30
	MPH	IGFBP3	TMEM219	0.00	0.00	0.01	0.58	0.45
	MPH	LGALS3	MERTK	0.00	0.00	0.01	0.27	0.62
	MPH	PPIA	BSG	0.00	0.00	0.00	0.89	0.24
	MPH	RARRES2	CCRL2	0.00	0.00	0.01	0.29	0.60
	MPH	RNASET2	TLR8	0.00	0.00	0.05	0.43	0.30
	MPH	SEMA3F	NRP2	0.00	0.00	0.04	0.29	0.39
	MPH	TNFSF10	TNFRSF10A	0.00	0.00	0.00	0.47	0.84
	MPH	UBASH3B	ESR1	1.00	1.00	0.01	0.24	0.60

Table S2.3. Significant L-R interactions between major cell categories in *Monodelphis domestica* 7.5dpc. Secreted signaling only.

source	target	ligand_complex	receptor_complex	cellphone_pvals	specificity_rank	magnitude_rank	ligand_%_exp	receptor_%_exp
ESF-SMOC2	LE-OAT	HDGF	NCL	0.03	0.01	0.03	0.29	0.53
	LE-OAT	IGF1	IGFBP3	0.00	0.00	0.00	0.84	1.00
	LE-OAT	IGFBP3	TMEM219	0.00	0.00	0.00	0.84	0.26
	LE-OAT	PPIA	BSG	0.00	0.00	0.00	0.86	0.59
	LE-SLCO2A1	BMP2	ACVR2A_BMPR1B	0.00	0.00	0.02	0.49	0.27
	LE-SLCO2A1	BMP2	BMPR1B_BMPR2	0.00	0.00	0.02	0.49	0.22
	LE-SLCO2A1	GPI	AMFR	1.00	1.00	0.08	0.23	0.30
	LE-SLCO2A1	HDGF	NCL	1.00	1.00	0.11	0.29	0.25
	LE-SLCO2A1	HGF	MET	0.00	0.00	0.01	0.34	0.48
	LE-SLCO2A1	IGF1	IGF1R	0.00	0.00	0.00	0.84	0.22
	LE-SLCO2A1	IGF1	IGFBP3	0.00	0.00	0.00	0.84	0.97
	LE-SLCO2A1	PPIA	BSG	0.00	0.00	0.00	0.86	0.58
	MPH	APP	SORL1	0.00	0.00	0.01	0.68	0.52
	MPH	APP	TNFRSF21	0.00	0.00	0.02	0.68	0.25
	MPH	CXCL12	CXCR4	0.00	0.00	0.08	0.31	0.31
	MPH	CXCL14	CXCR4	0.00	0.00	0.06	0.36	0.31
	MPH	GAS6	MERTK	0.00	0.00	0.01	0.49	0.64
	MPH	GPI	AMFR	1.00	1.00	0.06	0.23	0.49
	MPH	HDGF	NCL	0.00	0.00	0.02	0.29	0.65
	MPH	IGF1	IGF1R	0.00	0.00	0.00	0.84	0.64
	MPH	IGF1	IGFBP3	0.00	0.00	0.00	0.84	0.47
	MPH	IGFBP3	TMEM219	0.00	0.00	0.00	0.84	0.37
	MPH	LGALS3	MERTK	0.00	0.00	0.00	0.65	0.64
	MPH	PPIA	BSG	0.00	0.15	0.00	0.86	0.35
	MPH	PTGES3	PTGER4	0.94	0.63	0.02	0.77	0.23
	MPH	RARRES2	CCRL2	0.00	0.00	0.00	0.80	0.34
	MPH	RNASET2	TLR8	0.00	0.00	0.03	0.46	0.49
	MPH	SEMA3C	NRP2	0.00	0.00	0.01	0.48	0.51
	MPH	SULT1A1	PPARD	0.00	0.00	0.06	0.38	0.34
	MPH	SULT1A1	PPARG	0.00	0.00	0.01	0.38	0.63
	MPH	TNFSF10	TNFRSF10A	0.00	0.00	0.00	0.23	0.73
	MPH	UBASH3B	ESR1	1.00	1.00	0.06	0.26	0.37
	MPH	VEGFA	FLT1	0.99	1.00	0.09	0.33	0.25
	MPH	VEGFA	NRP2	0.00	0.01	0.02	0.33	0.51
LE-OAT	ESF-SMOC2	ALDH1A1	CRABP2_RARB	0.00	0.00	0.16	0.22	0.28
	ESF-SMOC2	ALDH1A1	CRABP2_RXRA	0.00	0.00	0.16	0.22	0.28
	ESF-SMOC2	ALDH1A1	RORB	0.00	0.00	0.04	0.22	0.48
	ESF-SMOC2	BDNF	NGFR	0.00	0.00	0.11	0.31	0.23
	ESF-SMOC2	BDNF	NTRK2	0.00	0.00	0.02	0.31	0.52
	ESF-SMOC2	FTH1	SCARA5	0.00	0.00	0.00	1.00	0.61
	ESF-SMOC2	FTL	SCARA5	0.00	0.00	0.00	1.00	0.61
	ESF-SMOC2	GPI	AMFR	0.21	0.28	0.04	0.27	0.63
	ESF-SMOC2	HDGF	NCL	0.00	0.00	0.02	0.35	0.68
	ESF-SMOC2	IGFBP3	TMEM219	0.00	0.00	0.00	1.00	0.21
	ESF-SMOC2	LGALS3	MERTK	0.00	0.00	0.00	0.81	0.44
	ESF-SMOC2	PGF	NRP1	0.00	0.00	0.01	0.61	0.36
	ESF-SMOC2	PGF	NRP2	0.00	0.00	0.02	0.61	0.27
	ESF-SMOC2	PPIA	BSG	0.00	0.00	0.00	1.00	0.44
	ESF-SMOC2	SRD5A2	AR	0.00	0.00	0.09	0.25	0.39
	ESF-SMOC2	VEGFA	NRP1	0.00	0.01	0.05	0.31	0.36
	ESF-SMOC2	VEGFA	NRP2	0.89	1.00	0.09	0.31	0.27
	ESF-SMOC2	WNT11	SFRP1	0.00	0.00	0.10	0.31	0.27
	VEC	ADM2	CALCRL_RAMP2	0.00	0.00	0.00	0.47	0.67
	VEC	GPI	AMFR	0.79	0.14	0.06	0.27	0.38
	VEC	HDGF	NCL	0.00	0.00	0.02	0.35	0.51
	VEC	IGFBP3	TMEM219	0.00	0.00	0.00	1.00	0.20
	VEC	PGF	FLT1	0.00	0.00	0.00	0.61	1.00
	VEC	PGF	FLT1_KDR	0.00	0.00	0.00	0.61	0.71
	VEC	PGF	NRP1	0.00	0.00	0.00	0.61	0.69
	VEC	PGF	NRP2	0.00	0.00	0.00	0.61	0.55
	VEC	PPIA	BSG	0.00	0.00	0.00	1.00	0.24
	VEC	VEGFA	FLT1	0.00	0.00	0.00	0.31	1.00

source	target	ligand_complex	receptor_complex	cellphone_pvals	specificity_rank	magnitude_rank	ligand_%_exp	receptor_%_exp
	VEC	VEGFA	FLT1_KDR	0.00	0.00	0.00	0.31	0.71
	VEC	VEGFA	KDR	0.00	0.00	0.00	0.31	0.71
	VEC	VEGFA	NRP1	0.00	0.00	0.00	0.31	0.69
	VEC	VEGFA	NRP2	0.00	0.00	0.01	0.31	0.55
	VEC	WNT11	FZD6_LRP6	0.00	0.00	0.04	0.31	0.26
LE-SLCO2A1	**ESF-SMOC2**	APP	SORL1	0.99	1.00	0.04	0.30	0.42
	ESF-SMOC2	APP	TNFRSF21	0.14	0.02	0.05	0.30	0.30
	ESF-SMOC2	BDNF	NGFR	0.00	0.00	0.11	0.23	0.23
	ESF-SMOC2	BDNF	NTRK2	0.00	0.00	0.02	0.23	0.52
	ESF-SMOC2	FGF1	FGFR1	0.00	0.00	0.05	0.32	0.22
	ESF-SMOC2	FTH1	SCARA5	0.00	0.00	0.00	0.99	0.61
	ESF-SMOC2	FTL	SCARA5	0.00	0.00	0.00	0.57	0.61
	ESF-SMOC2	IGFBP3	TMEM219	0.00	0.00	0.00	0.97	0.21
	ESF-SMOC2	LGALS3	MERTK	0.00	0.00	0.03	0.27	0.44
	ESF-SMOC2	PGF	NRP1	0.00	0.00	0.02	0.40	0.36
	ESF-SMOC2	PGF	NRP2	0.00	0.00	0.03	0.40	0.27
	ESF-SMOC2	PPIA	BSG	1.00	1.00	0.01	0.54	0.44
	ESF-SMOC2	PTPRD	IL1RAP	0.00	0.00	0.08	0.27	0.23
	ESF-SMOC2	SRD5A2	AR	0.00	0.00	0.00	0.74	0.39
	ESF-SMOC2	WNT11	SFRP1	0.00	0.00	0.02	0.44	0.27
	VEC	ADM2	CALCRL_RAMP2	0.00	0.00	0.01	0.21	0.67
	VEC	IGFBP3	TMEM219	0.00	0.00	0.00	0.97	0.20
	VEC	PGF	FLT1	0.00	0.00	0.00	0.40	1.00
	VEC	PGF	FLT1_KDR	0.00	0.00	0.00	0.40	0.71
	VEC	PGF	NRP1	0.00	0.00	0.00	0.40	0.69
	VEC	PGF	NRP2	0.00	0.00	0.01	0.40	0.55
	VEC	PPIA	BSG	1.00	1.00	0.01	0.54	0.24
	VEC	PTPRD	IL1RAP	0.00	0.00	0.05	0.27	0.22
	VEC	WNT11	FZD6_LRP6	0.00	0.00	0.01	0.44	0.26
MPH	**ESF-SMOC2**	ALDH1A1	CRABP2_RARB	0.00	0.00	0.09	0.35	0.28
	ESF-SMOC2	ALDH1A1	CRABP2_RXRA	0.00	0.00	0.09	0.35	0.28
	ESF-SMOC2	ALDH1A1	RORB	0.00	0.00	0.02	0.35	0.48
	ESF-SMOC2	APP	SORL1	1.00	1.00	0.10	0.20	0.42
	ESF-SMOC2	APP	TNFRSF21	1.00	1.00	0.12	0.20	0.30
	ESF-SMOC2	CXCL2	DPP4	0.45	0.35	0.02	0.34	0.47
	ESF-SMOC2	CXCL9	DPP4	0.02	0.00	0.03	0.24	0.47
	ESF-SMOC2	FTH1	SCARA5	0.00	0.00	0.00	1.00	0.61
	ESF-SMOC2	FTL	SCARA5	0.00	0.00	0.00	0.75	0.61
	ESF-SMOC2	GPI	AMFR	0.00	0.00	0.03	0.38	0.63
	ESF-SMOC2	HGF	MET	0.89	0.04	0.10	0.28	0.20
	ESF-SMOC2	IGFBP3	TMEM219	1.00	1.00	0.07	0.47	0.21
	ESF-SMOC2	IL1A	IL1R1_IL1RAP	0.00	0.00	0.02	0.21	0.23
	ESF-SMOC2	IL1A	IL1R2_IL1RAP	0.00	0.00	0.00	0.21	0.23
	ESF-SMOC2	IL1B	IL1R1_IL1RAP	0.00	0.00	0.00	0.45	0.23
	ESF-SMOC2	IL1B	IL1R2_IL1RAP	0.00	0.00	0.00	0.45	0.23
	ESF-SMOC2	IL1RN	IL1R1_IL1RAP	0.00	0.00	0.00	0.53	0.23
	ESF-SMOC2	IL1RN	IL1R2_IL1RAP	0.00	0.00	0.00	0.53	0.23
	ESF-SMOC2	LGALS3	MERTK	0.00	0.00	0.02	0.49	0.44
	ESF-SMOC2	LIPA	RORA	0.00	0.00	0.00	0.37	0.86
	ESF-SMOC2	PDGFC	PDGFRA	0.00	0.00	0.00	0.61	0.92
	ESF-SMOC2	PPIA	BSG	0.00	0.01	0.00	0.82	0.44
	ESF-SMOC2	RARRES2	CCRL2	0.00	0.00	0.06	0.41	0.32
	ESF-SMOC2	TGFB1	ACVR1_TGFBR2	0.00	0.00	0.03	0.42	0.47
	ESF-SMOC2	TGFB1	TGFBR3	0.00	0.00	0.01	0.42	0.72
	ESF-SMOC2	UBASH3B	ESR1	0.00	0.00	0.00	0.53	0.98
	ESF-SMOC2	VEGFA	NRP1	0.00	0.02	0.05	0.31	0.36
	ESF-SMOC2	VEGFA	NRP2	1.00	1.00	0.10	0.31	0.27
	VEC	CXCL2	DPP4	0.79	0.09	0.03	0.34	0.25
	VEC	CXCL9	DPP4	0.33	0.01	0.04	0.24	0.25
	VEC	GPI	AMFR	0.17	0.01	0.04	0.38	0.38
	VEC	HGF	MET	0.06	0.00	0.05	0.28	0.31

source	target	ligand_complex	receptor_complex	cellphone_pvals	specificity_rank	magnitude_rank	ligand_%_exp	receptor_%_exp
	VEC	IGFBP3	TMEM219	1.00	1.00	0.06	0.47	0.20
	VEC	IL1A	IL1R1_IL1RAP	0.00	0.00	0.10	0.21	0.25
	VEC	IL1B	IL1R1_IL1RAP	0.00	0.00	0.02	0.45	0.25
	VEC	IL1RN	IL1R1_IL1RAP	0.00	0.00	0.01	0.53	0.25
	VEC	PPIA	BSG	0.00	0.27	0.00	0.82	0.24
	VEC	RARRES2	CCRL2	0.00	0.00	0.04	0.41	0.29
	VEC	TGFB1	ACVR1_TGFBR2	0.00	0.00	0.05	0.42	0.26
	VEC	TGFB1	TGFBR3	0.00	0.00	0.03	0.42	0.42
	VEC	VEGFA	FLT1	0.00	0.00	0.00	0.31	1.00
	VEC	VEGFA	FLT1_KDR	0.00	0.00	0.00	0.31	0.71
	VEC	VEGFA	KDR	0.00	0.00	0.00	0.31	0.71
	VEC	VEGFA	NRP1	0.00	0.00	0.00	0.31	0.69
	VEC	VEGFA	NRP2	0.00	0.00	0.01	0.31	0.55
VEC	**LE-OAT**	IGFBP3	TMEM219	0.00	0.00	0.00	0.75	0.26
	LE-OAT	PPIA	BSG	0.00	0.00	0.00	0.83	0.59
	LE-SLCO2A1	GPI	AMFR	0.91	0.12	0.06	0.25	0.30
	LE-SLCO2A1	HBEGF	EGFR	0.00	0.00	0.00	0.56	0.98
	LE-SLCO2A1	HBEGF	ERBB4	0.00	0.00	0.00	0.56	0.96
	LE-SLCO2A1	PPIA	BSG	0.00	0.00	0.00	0.83	0.58
	MPH	APP	SORL1	0.00	0.00	0.01	0.44	0.52
	MPH	APP	TNFRSF21	0.01	0.01	0.04	0.44	0.25
	MPH	GAS6	MERTK	0.00	0.00	0.01	0.25	0.64
	MPH	GPI	AMFR	0.44	0.34	0.04	0.25	0.49
	MPH	IGFBP3	TMEM219	0.00	0.00	0.00	0.75	0.37
	MPH	PPIA	BSG	0.00	0.01	0.00	0.83	0.35
	MPH	PTGES3	PTGER4	1.00	1.00	0.03	0.55	0.23
	MPH	RNASET2	TLR8	0.00	0.00	0.05	0.20	0.49
	MPH	SEMA3F	NRP2	0.00	0.00	0.02	0.34	0.51
	MPH	TNFSF10	TNFRSF10A	0.00	0.00	0.00	0.31	0.73

Table S2.4. Significant L-R interactions between major cell categories in *Monodelphis domestica* 13.5dpc. Secreted signaling only.

Mice			Guinea pig			
LAST_top_orthold	DSC_fmi	ESF_imp	LAST_top_orthold	dS3-PRL_fmi	dS3-OXT_fmi	ESF_imp
ADM	1	1	ADM	1	1	0
AGRN	1	1	AGRN	1	1	0
AGRP	0	1	ANGPT1	0	0	1
ANGPT1	0	1	ANGPT4	0	1	0
ANGPT2	1	0	ANXA1	1	1	1
ANGPT4	1	0	APLP2	1	1	1
ANXA1	1	1	APOA1	1	1	0
APELA	0	1	APOE	1	1	1
APLP2	1	1	APP	1	1	1
APOA1	1	0	B2M	1	1	1
APOA2	1	0	BAG6	1	1	1
APOB	1	0	BDNF	0	1	0
APOE	1	1	BMP2	1	1	0
APP	1	1	BMPR2	1	1	1
ASIP	0	1	C3	0	0	1
BAG6	1	1	C4A	0	0	1
BMP4	0	1	CCL11	0	0	1
BMP7	0	1	CLU	1	1	1
BMP8A	1	0	CSF2	1	1	0
BMPR2	1	1	CXCL12	1	1	1
BST2	1	1	CXCL14	1	1	0
C3	0	1	CXCL2	1	1	0
C4A	0	1	CXCL8	1	0	0
CCL11	0	1	DKK2	1	1	1
CCL25	0	1	EDN3	0	0	1
CLU	0	1	ENTPD1	0	0	1
CSF1	0	1	F2	1	1	1
CXCL10	0	1	FGF1	0	0	1
CXCL12	0	1	FGF2	0	0	1
CXCL14	1	1	FGF7	1	1	1
CXCL16	0	1	FTH1	1	1	1
DKK2	0	1	FTL	1	1	1
EDN3	0	1	GAS6	0	1	1
ENTPD1	0	1	GDF11	0	0	1
FGF10	0	1	GPI	1	1	1
FGF2	1	1	HBEGF	1	0	0
FGF7	0	1	HDGF	1	1	1
FTH1	1	1	HEBP1	1	1	1
FTL	1	1	HFE	0	0	1
GAS6	0	1	HGF	0	0	1
GDF10	0	1	IGF1	1	1	1
GDF11	0	1	IGF2	1	0	1
GDF7	0	1	IGFBP3	1	1	1
GHRH	1	0	IL15RA	1	1	1
GPI	1	1	IL16	1	1	1
HBEGF	1	0	IL18	0	1	0
HDGF	1	1	IL34	0	1	0

HEBP1	0	1	**INHBB**	0	1	0
HFE	1	1	**JAG1**	0	0	1
HGF	0	1	**KITLG**	1	1	1
IGF1	0	1	**LCN2**	1	0	0
IGFBP3	0	1	**LGALS3**	1	1	1
IL15	0	1	**LTB**	0	0	1
IL15RA	0	1	**MDK**	0	0	1
IL16	0	1	**MMP2**	1	1	1
IL18	0	1	**NDP**	0	0	1
IL33	0	1	**NMB**	1	0	0
INHBA	1	0	**NPY**	1	1	1
INHBB	1	0	**NTF3**	0	0	1
JAG1	0	1	**OXT**	1	1	0
KNG1	0	1	**PDGFC**	0	1	1
LEFTY1	0	1	**PDGFD**	1	1	1
LGALS3	1	1	**PENK**	1	1	1
MDK	0	1	**PGF**	0	1	1
MMP2	1	1	**PPIA**	1	1	1
NDP	0	1	**PRL**	1	0	0
NPB	1	0	**PROK2**	1	0	0
NSMF	0	1	**PSAP**	1	1	1
PDGFA	1	0	**PTN**	0	0	1
PDGFC	0	1	**RARRES2**	0	0	1
PDGFD	1	1	**RNASET2**	1	1	1
PENK	0	1	**RSPO3**	0	0	1
PGF	1	0	**SEMA3A**	1	1	1
PI16	0	1	**SEMA3C**	1	1	1
PLAU	1	1	**SEMA3E**	0	0	1
PPIA	1	1	**SEMA3F**	0	0	1
PRL	1	0	**TAFA5**	0	0	1
PSAP	1	1	**TAX1BP3**	1	1	1
PTN	1	1	**TF**	0	0	1
PTPRD	0	1	**TGFB1**	1	1	1
RARRES2	1	1	**TGFB2**	0	1	1
RBP4	1	1	**TGM2**	1	1	1
RNASET2	1	1	**TNFSF10**	1	1	1
RSPO1	0	1	**TNFSF12**	0	0	1
RSPO3	1	1	**VEGFA**	1	1	1
SAA1	0	1	**VEGFB**	1	1	1
SCT	1	0	**VEGFC**	1	1	1
SEMA3A	0	1	**VEGFD**	0	0	1
SEMA3C	0	1	**VWF**	0	1	0
SEMA3E	1	0	**WNT2**	0	1	1
TAC3	0	1	**WNT5A**	1	1	1
TAFA5	0	1	**WNT6**	0	0	1
TAX1BP3	1	1	**WNT7A**	1	0	0
TF	1	1				
TGFA	1	0				
TGFB1	1	1				

TGFB2	1	1				
TGFB3	1	1				
TGM2	1	1				
TNFSF12	1	1				
TNFSF13	1	1				
TNFSF13B	0	1				
TNFSF9	1	1				
VEGFA	1	1				
VEGFB	1	1				
VEGFC	0	1				
VEGFD	1	1				
WNT16	0	1				
WNT4	1	1				
WNT5A	1	1				
WNT5B	0	1				
WNT9A	0	1				

Table S3. Conserved ligand expression between the decidua and stromal fibroblast in *Mus musculus* (days 15.5 and 4.5) and *Cavia porcellus* (days 30.5 and 6.5). DSD= decidua, ESF = endometrial stromal fibroblast.

Mice			Guinea pig			
BLAST_top_ortholog	TGCi_fmi	eEpi_imp	BLAST_top_ortholog	EPT_fmi	CTB_fmi	eEpi_imp
ADM	0	1	ADM	1	1	0
AGRN	1	1	AGRN	1	1	0
ANGPT2	1	1	ANGPT1	0	1	0
ANXA1	1	1	ANGPT2	0	0	1
APLP2	1	1	ANXA1	1	1	1
APOA1	1	0	APLP2	1	1	1
APOA2	1	0	APOA1	1	1	1
APOB	1	0	APOE	1	1	1
APOE	1	1	APP	1	1	1
APP	1	1	B2M	1	1	1
ASIP	1	0	BAG6	1	1	1
BAG6	1	1	BMP2	1	0	0
BMP7	0	1	BMP5	0	0	1
BMP8A	1	0	BMPR1B	0	0	1
BMPR1B	0	1	BMPR2	1	1	1
BMPR2	1	1	CCK	0	0	1
BST2	1	1	CCL15	1	0	0
BTC	0	1	CCL2	1	0	1
C3	0	1	CCL21	0	0	1
C4A	0	1	CLU	1	1	1
CALCA	0	1	CXCL12	1	1	1
CCL13	0	1	CXCL14	1	1	0
CCL23	0	1	CXCL16	0	1	1
CCL25	1	1	CXCL8	1	0	0
CCL28	0	1	DKK2	1	1	0
CCL4	0	1	EREG	0	1	0
CLCF1	0	1	F2	1	1	0
CLU	1	1	FGF7	1	0	0
CSF1	1	1	FTH1	1	1	1
CX3CL1	0	1	FTL	1	1	1
CXCL12	0	1	GDF11	0	0	1
CXCL14	0	1	GDF15	0	1	0
CXCL16	0	1	GPI	1	1	1
CXCL2	0	1	HBEGF	0	1	0
EDN1	0	1	HDGF	1	1	1
ENTPD1	1	0	HEBP1	1	1	1
FGL1	0	1	IGF1	1	1	1
FTH1	1	1	IGFBP3	1	1	1
FTL	1	1	IHH	0	0	1
GAS6	0	1	IL34	1	0	0
GDF10	0	1	INHBB	0	1	1
GDF15	0	1	JAG1	0	1	1
GHRH	1	0	KITLG	1	1	1
GPI	1	1	LCN2	1	1	1
HBEGF	1	1	LEP	1	0	0

HDGF	1	1	**LGALS3**	1	1	1
HEBP1	1	1	**LTB**	0	0	1
HFE	1	1	**MDK**	0	1	1
IGF1	0	1	**MMP2**	1	1	1
IGF2	1	0	**NMB**	1	1	1
IGFBP3	1	1	**NPY**	1	1	0
IHH	0	1	**NTF3**	0	0	1
IL15	0	1	**OXT**	1	0	0
IL15RA	0	1	**PDGFC**	0	1	1
IL18	0	1	**PDGFD**	0	0	1
IL34	0	1	**PENK**	1	1	1
INHBB	1	1	**PGF**	0	0	1
JAG1	1	1	**POMC**	0	0	1
KISS1	0	1	**PPIA**	1	1	1
KITLG	1	1	**PROK1**	0	0	1
KNG1	0	1	**PROK2**	0	1	1
LCN2	0	1	**PSAP**	1	1	1
LGALS3	1	1	**PTH2**	0	1	0
LIF	0	1	**PTN**	0	0	1
MDK	1	1	**RARRES2**	0	0	1
MMP2	0	1	**RNASET2**	1	1	1
NMB	1	1	**RSPO3**	0	0	1
NRTN	0	1	**SAA1**	0	1	1
NSMF	1	1	**SEMA3A**	1	0	0
PDGFA	1	1	**SEMA3C**	1	1	1
PDGFC	0	1	**SEMA3E**	0	0	1
PDGFD	1	1	**SEMA3F**	0	0	1
PENK	0	1	**TAX1BP3**	1	1	1
PGF	1	1	**TF**	0	0	1
PI16	1	1	**TGFA**	0	1	1
PPBP	0	1	**TGFB1**	1	1	1
PPIA	1	1	**TGFB2**	0	1	1
PRL	1	1	**TGM2**	1	1	1
PROC	1	0	**TNFSF10**	1	1	1
PSAP	1	1	**TNFSF13**	0	0	1
PTN	1	1	**VEGFA**	0	1	1
PTPRD	1	1	**VEGFB**	1	1	1
RARRES2	1	1	**VEGFC**	1	0	1
RBP4	0	1	**VWF**	1	1	1
RNASET2	1	1	**WNT11**	0	0	1
RSPO1	0	1	**WNT2**	0	0	1
SCT	1	0	**WNT5A**	0	0	1
SEMA3A	0	1	**WNT7B**	0	0	1
SEMA3C	0	1				
SEMA3E	1	1				
SEMA3F	1	1				

TAX1BP3	1	1
TF	1	1
TGFA	0	1
TGFB1	1	1
TGFB2	1	1
TGFB3	1	1
TGM2	1	1
THPO	0	1
TNFSF10	0	1
TNFSF12	0	1
TNFSF13	0	1
VEGFA	1	1
VEGFB	1	1
VEGFD	0	1
WNT11	0	1
WNT4	0	1
WNT5A	0	1

Table S4.1. Conserved ligand expression between the trophoblast and maternal epithelium in *Mus musculus* (days and 15.5 and 4.5) and *Cavia porcellus* (days 30.5 and 6.5).
TGCi = invasive trophoblast giant cells, eEpi = endometrial epithelium, EPT = extraplacental trophoblast, CTB = cytotrophoblast

Opossum				Human		
BLAST_top_ortholog	CTB_LG	SCT_LG	eEpi_MG	external_gene_name	EPT_fmi	eEpi_Secretory
AGRN	1	1	1	ADM	1	1
ANXA1	1	1	1	AGRN	1	1
APLP2	1	1	1	ANGPT1	1	0
APOA1	1	0	0	ANGPT2	1	0
APOA2	1	1	0	ANGPT4	1	0
APOB	1	0	0	ANXA1	1	1
APOE	1	1	1	APLP2	1	1
APP	1	1	1	APOE	1	1
AREG	1	0	0	APP	1	1
BAG6	1	1	1	B2M	1	1
BMP2	1	0	1	BAG6	1	0
BMP4	1	1	0	BMP4	1	0
BMP6	1	0	0	BMP7	1	0
BMP7	0	1	0	BMPR1B	0	1
BMP8A	1	0	0	BMPR2	1	1
BMPR1B	1	0	1	BST2	1	1
BMPR2	1	1	1	C3	0	1
C4A	1	1	1	CCL20	0	1
CCL14	0	1	0	CCL28	0	1
CLU	1	1	1	CCL4	0	1
CXCL14	0	1	0	CGA	1	0
CXCL16	1	1	0	CLCF1	0	1
CXCL2	0	1	0	CLU	1	1
CXCL8	0	1	0	CSF1	0	1
EDN1	1	0	0	CSH1	1	0
EDN3	1	0	0	CSH2	1	0
FGF1	1	0	0	CXCL14	1	1
FGF8	0	1	0	CXCL16	0	1
FTH1	1	1	1	CXCL2	0	1
FTL	1	1	1	CXCL3	0	1
GAS6	0	1	0	CXCL8	0	1
GPI	1	1	1	EBI3	1	0
HBEGF	1	1	0	EDN1	0	1
HDGF	1	0	1	ENTPD1	1	0
HEBP1	0	1	0	ERVH48-1	1	0
IGF1	0	0	1	FGF7	0	1
IGFBP3	1	1	0	FTH1	1	1
IHH	0	0	1	FTL	1	1
IL10	0	1	0	GAL	1	0
IL15	0	0	1	GAS6	0	1
IL17A	0	1	0	GAST	0	1
IL1A	0	1	0	GDF11	0	1
IL6	0	1	0	GDF15	1	1
JAG1	1	0	0	GH2	1	0
JAG2	1	1	1	GPI	1	1

LCN2	0	1	0
LGALS3	1	1	0
MMP2	0	1	1
MSTN	0	0	1
NMB	0	0	1
NRTN	0	1	0
NTF3	1	0	1
PDGFA	0	1	0
PDGFC	1	0	0
PPIA	1	1	1
PSAP	0	1	1
PTPRD	1	0	0
RBP4	1	1	0
REN	0	0	1
RLN2	0	1	0
RNASET2	1	1	1
RNLS	1	1	1
SEMA3A	0	1	1
SEMA3C	1	0	0
SEMA3E	1	0	0
TGFA	1	1	1
TGFB1	1	1	0
TGFB2	1	0	0
TGM2	1	0	1
VEGFA	1	1	1
VEGFB	0	0	1
WNT3A	0	1	0
WNT4	1	1	0
WNT5B	1	0	1
WNT6	0	1	0
WNT7B	0	1	0
WNT9A	1	1	0

HBEGF	0	1
HDGF	1	1
HEBP1	1	1
IGF1	0	1
IGF2	1	0
IGFBP1	1	1
IGFBP3	1	1
IHH	0	1
IL18	0	1
IL1A	0	1
IL1RN	0	1
IL6	0	1
IL7	1	0
INHA	1	0
INHBA	1	0
INHBB	0	1
JAG1	0	1
JAG2	0	1
KISS1	1	0
LCN2	0	1
LEFTY1	0	1
LEP	1	0
LGALS3	1	1
LGALS9	0	1
LIF	0	1
MDK	1	1
MMP2	1	0
MST1	1	0
NMB	1	1
NSMF	0	1
PAEP	1	1
PDGFA	0	1
PGF	1	0
PLAU	0	1
PPIA	1	1
PSAP	1	1
PTN	1	1
RBP4	1	1
RNASET2	1	1
SAA1	1	0
SEMA3C	0	1
SEMA3E	0	1
SHH	0	1
SPX	0	1
TAX1BP3	1	1
TGFA	0	1

TGFB1	1	0
TGFB2	1	1
TGM2	1	1
TNFSF10	1	1
TNFSF12	0	1
TNFSF13	0	1
TNFSF15	0	1
TNFSF9	0	1
VEGFA	0	1
VEGFB	1	1
WNT2B	1	0
WNT7A	1	0
WNT7B	0	1

Table S4.2. Conserved ligand expression between the trophoblast and maternal epithelium in *Monodelphis domestica* (days and 13.5 and 7.5) and *Homo sapiens* (first trimester and mid-secretory).

CONCLUDING DISCUSSION

I would like to re-address this thesis's main findings and claims, along with their implications and prospects for the field of female reproductive biology and the study of pregnancy.

1. *Progesterone did not first evolve for its current function in placental mammals.*

The corpus luteum is an endocrine gland with an intrinsic lifespan susceptible to co-option and responsible for progesterone production. To trace the co-option of ovarian progesterone during gestation, I wanted to grasp the evolutionary context in which the corpus luteum's progesterone production first evolved in vertebrates. Ovarian progesterone is linked to mammalian reproduction, determining gestation length. However, the corpus luteum and the progesterone hormone originated long before viviparity and even before internal fertilization. This fact motivated me to reconsider the reproductive role and adaptive significance we unequivocally attach to its origin. In the first chapter of my thesis, I considered two main models to trace the evolution of progesterone's first function. The first is the hypothesis that estrogen (and hence the intermediate progesterone) first originated as a byproduct of the synthesis and degradation of yolks and cholesterol in follicular cells (Browning, 1973). The second is the ligand exploitation model, according to which progesterone was selected even before the origin of its signaling functions through the progesterone receptor (Thornton, 2001). Emerging first as a catabolic byproduct and selected as an intermediate steroid for the early functions of estrogen, I have argued that counteracting vitellogenesis was probably progesterone's ancestral function, followed by a role in inhibiting folliculogenesis and spacing the time to the next ovulation, i.e., prompting the origin of a luteal phase in the ovarian cycle.

Furthermore, ovariectomies during pregnancy - i.e., surgical removal of the corpus luteum - have profound physiological consequences in placental mammals but not always in non-mammalian vertebrates, suggesting that there is no universal requirement for progesterone outside of placentals. I have shown why, in non-mammalian species, progesterone is only essential in specific stages of gestation. Multiple roles of progesterone probably evolved with the independent evolution and numerous origins of internal fertilization and viviparity. These derived functions include the coordination between the ovary and the oviduct or endometrium, regulating the pace of embryonic development, enhancing uterine histotrophic secretions, complementing lecithotrophic provisioning, and inhibiting myometrial contractions.

2. *The luteal phase can constrain the evolution of a longer gestation length.*

I found no definitive evidence for exogenous regulation to increase corpus luteum lifespan during pregnant cycles in non-mammalian vertebrates, which supports the thesis that trans-cyclic gestation (Chavan et al., 2016) or pregnancy longer than a sterile luteal phase constitutes a significant innovation of eutherian mammals. This innovation entails two hallmarks with broad consequences. First, the transfer of endocrine control from the mother to the blastocyst and, later in pregnancy, to placental sources of steroidogenesis. Second, this punctuated change allowed the decoupling of pregnant from non-pregnant cycles, enabling the independent extension of the latter.

Repeated, non-fertile cycles constitute an oddity under natural, wild conditions. Based on this, one could downplay the selective forces and physiological constraints associated with the length of endogenous ovarian cycles and write it off as an artifact (Conaway, 1971; Hayssen, 2020). In this first chapter, I hope to show that the non-pregnant cycle length and corpus luteum lifespan are actively evolving traits that have consequences for the evolution of pregnancy. Our data on eutherian

reproduction indicates that the non-pregnant cycle length does act as an evolutionary constraint for the variation of gestation length, as the two do not vary completely independently. This observation demonstrates the importance of understanding the non-pregnant cycle and its physiological connection to the evolution of pregnancy, an insight that can motivate future research.

3. *Gestation length is not a unitary, homologous trait in eutherians.*

An enormous variety of mechanisms controlling gestational progesterone across eutherians is evident. Along with the lack of available information for many wild species, this diversity further complicates an inference on the ancestral cycle and gestation length and the understanding of possible ancestral and derived patterns. Instead of a singular event in decoupling the length between a non-pregnant and pregnant cycle (i.e., the non-pregnant luteal length remaining the same and the pregnant one increasing), different and independent events involving different scenarios have arisen within the eutherian lineages. These scenarios include shortening only the non-pregnant cycle, shortening the non-pregnant cycle while extending the pregnant cycle, and extending both cycles simultaneously. I hope to have convincingly shown how this aligns with the lack of conservation in prolonging gestation, from the mechanism of maternal recognition of pregnancy (maternal, anti-luteolytic, or pro-luteolytic) to the sources of placental progesterone. These different ways of changing gestational length relative to the luteal phase underlie the extensive variation in gestation length found in eutherians compared to other higher taxonomic groups. This view has consequences for studying the evolution of pregnancy and gestation length in eutherians. In particular, it has implications for comparative analysis of pregnancy across eutherian species.

4. *Phylogenetic state reconstructions can easily overestimate the ancestral length of eutherian gestation.*

One of the consequences of the independent evolution of later stages of gestation across lineages is that, most likely, a very long gestation did not evolve only once, nor was ancestral in eutherians, particularly when we consider the relatively small size of the eutherian ancestor (O'Leary et al., 2013). Reproductive traits, from gestation length to placental structure (Martin & MacLarnon, 1985; Lewitus & Soligo, 2011), are uniquely connected to life history traits such as body mass and longevity. I have argued that eutherian gestation is a serial homolog to the luteal phase and that the origin of eutherian maternal recognition of pregnancy released a variational constraint to modify gestation length, mainly driven by changes in body size, without affecting the ovarian cycle. Therefore, based on current body size and gestation length, phylogenetic inferences likely tend to overestimate the ancestral gestation length.

5. *Understanding the evolution of female reproduction entails studying traits beyond its reproductive function.*

Throughout the first part of my thesis, I have highlighted the interest in studying reproductive traits through their whole arch of evolution to better understand ancestral and derived functions. This includes studying traits before they acquire reproductive functions and after these functions are lost. The second chapter of my thesis centers on a trait at the border of reproductive and non-reproductive functions: the female orgasm. The adaptive functions of female orgasm have long been contested. From the outset, I expected that the evolutionary debate on the function and evolutionary origin of the female orgasm would be influenced by biases that enclose sexuality in reproduction, particularly when it comes to conceptualizing the female body. During my analysis of evolutionary views on this trait,

however, I observed how this divide between sexuality and reproduction in different pre-evolutionary characterizations of the female orgasm translated into a debate between non-adaptationist and adaptationist approaches to the evolutionary origin of the trait. In particular, the discussion on whether the female orgasm has reproductive functions or not has taken place between evo-devo and adaptationist advocates. Tracing evolutionary explanations of the female orgasm illustrates the search for proof of a trait's adaptive significance even after the repeated failure to find one. These types of approaches neglect the importance of a trait beyond its reproductive role, as well as its dynamic changes.

6. *The concept of homology is an unifying thread to the scientific hypotheses on the female orgasm.*

The main argument I made throughout the second chapter is that differing assumptions of homology form a unifying thread of the representations or hypotheses on the female orgasm. Being aware of the variation and diversity in the scientific depiction of female sexuality across historical settings helped me to appreciate the continuity in how the concept of homology underpinned many of the scientific hypotheses on the female orgasm. I have analyzed how different concepts of homology have been deployed to formulate different research hypotheses on the female orgasm through various periods. These concepts include statements based on sexual, developmental, functional, and phylogenetic homology, and each has different implications and is influenced by a different context. My analysis puts into dialogue the different homology-based theories of the female orgasm. In doing so, I have also traced different historical forms of homology thinking.

7. *The transition to invasive placentation entailed the expansion of the trophoblast cell types, invasive properties, and a pre-decidual phenotype.*

In the third chapter of my thesis, I examined cell-cell communication inferred from single-cell transcriptomes to study the evolution of invasive placentation in eutherians. A significant discontinuity regarding cell-type composition across species was trophoblast diversity. We have argued that the therian ancestor had two trophoblast cell categories corresponding to the eutherian cytotrophoblast and syncytiotrophoblast, which aligns with the most fundamental trophoblast lineage divide. This resembles the situation in opossum, the marsupial outgroup used in the comparisons. The next discontinuity is the origin of invasive trophoblast populations in eutherians, which we have characterized already in the Afrotherian tenrec. Thus, we expect invasive trophoblast to be the universal characteristic of all invasive placentation.

The question of homology is uniquely challenging when it comes to the trophoblast. It remained to be seen which phenotypic distinctions, whether functional or morphological, better corresponded with the transcriptome. I annotated the trophoblast clusters guided by the presence of placental-specific genes, the expression of invasive genes, and knowledge of the placental region in which they were located. I used traditional criteria and previous definitions according to morphological features to designate glycogen trophoblast, giant trophoblast cells, or syncytiotrophoblast. Among all these features, invasiveness consistently stood out as the best predictor of transcriptomic similarity, and assortment by invasiveness made the most fundamental split across our data. Extraplacental trophoblasts of tenrec and guinea pig, extravillous trophoblast in the macaque and human, and spiral artery-remodeling and glycogen trophoblast in the mouse shared a common gene expression signature. The grouping of phylogenetically distant trophoblast transcriptomes by invasive properties and not by any other phenotypic characteristics suggests that the divide between invasive and non-invasive cell types was ancestral and may not only be due to convergent evolution. Across

species, transcriptomic similarity in invasive trophoblast populations was not only driven by the presence of effector genes, such as metalloproteinases and glycoproteins; transcription factors were also among the expressed genes underlying the high similarity scores. Given the breadth of the species we used, I predict that cells with the same topological position and invasive function would consistently share more transcriptomic similarity than cells sharing other aspects of the phenotype, like fusion or ploidy. If this holds when introducing additional species in the comparison, invasive populations could be *truly* homologous compared to the concurrent and independent acquisition of other phenotypes through endoreduplication or fusion.

Histological and descriptive work was an essential complement to single-cell data analysis. The morphological diversity in placental cells was more manifest in histology than in the single-cell analysis, making the need to complement single-cell studies with other, more traditional approaches more evident. In addition, we have applied new tools in single-cell analysis to address the origin and diversity of eutherian placental invasiveness. Fundamentally, these tools aim to identify homologous expression programs (Tarashansky 2019 et al., Tarashansky et al., 2021) and, therefore, emphasize similarities. Doing descriptive work to fully understand the anatomical regions and where different populations were predicted to be located was essential to ratify the results obtained from the transcriptomic data analysis.

Although it was expected that all species with hemochorial placentation have a clear pattern of decidualization, this had yet to be studied or confirmed. We have characterized a decidual cell type in the Afrotherian tenrec, even if previous histological studies have questioned its presence in this species (Carter et al., 2005). Based on this, we have proposed a stepwise model for the evolution of the decidua: the first step is from endometrial fibroblast to a pre-decidual cell type, with intermediate phenotype as found in tenrec, and the second step is to the typical secretory profile of the mature decidua, which we only found in Euarchontoglires.

This study is valuable for multiple reasons. For the first time, it includes single-cell data on non-model species, such as guinea pig and tenrec, along with descriptive and histological details. Knowledge of non-model species is essential in broadening our scope. By providing a comparative single-cell study of the placenta of different placental mammals, it tackles the question of cell type homology and evolution at the fetal-maternal interface for the first time by using the latest developments in the field.

8. Hallmarks of eutherian implantation were likely already present in the blastocyst stage of the therian ancestor.

I devoted the last chapter to exploring similarities and dissimilarities between mouse and guinea pig implantation and to investigating the evolutionary link to the equivalent processes in the marsupial opossum during two essential stages: the blastocyst stage and the “attachment” stage, when a brief cell-to-cell contact takes place between fetal and maternal tissues after hatching from the eggshell. The results presented in this chapter support two broad conclusions. The first one is the relative conservation of the peri-implantation period in distantly derived rodents, with main signatures at the single-cell and cell-cell communication levels underlying implantation conserved. The second is that eutherian implantation could have evolved from a blastocyst stage in the therian ancestor rather than post-hatching endometrial changes.

On the side of the maternal epithelium, while mouse and guinea pig share the same transcriptomic signature, we found that the opossum epithelium only expresses hallmark receptivity genes at the blastocyst stage. Likewise, main eutherian implantation signals were absent in the opossum epithelial-stromal crosstalk after the rupture of the eggshell but were present at the blastocyst stage.

I envisioned this chapter as a single-cell approach to the evolution of implantation as essential to the eutherian reproductive mode. However, including a marsupial species requires considering differences due to different reproductive strategies, which tend to be more flexible than the signatures underpinning reproductive modes and key reproductive traits such as implantation. Connected to this distinct reproductive strategy, the abundance and differential expression of glands were the most striking differences. Our results align with the hypothesis that suppressing estrogen signaling during implantation may be a derived attribute of eutherian reproduction and support the notion that inflammation could also play histotrophic roles in the opossum. On the side of the maternal stroma, whereas there was fibroblast proliferation in rodent peri-implantation, a drastic decline was found in the opossum as gestation proceeds, pointing out that the crucial role of the stroma during implantation is a derived, unique eutherian feature.

The intense interest in comparative studies of early embryos has generated data sets, which we used to infer communication between the pre-implantation blastocyst and the maternal tissue using our data, as we did for the placenta and uterus later in pregnancy. We found blastocyst gene expression of hatching proteases, Wnt, and BMP ligands conserved between the opossum and our two rodent taxa. Overall, our data suggest that maternal epithelial and stromal changes, rather than solely changes in the blastocyst, were vital to the evolution of eutherian implantation. Thus, we have argued that innovations and discontinuities in the maternal compartment were the key during the transition to eutherian implantation.

Embryo implantation is a critical reproductive process without which a long, sustained gestation cannot occur. I argue that besides resolving inflammation (Chavan et al., 2017), the presence of a shell throughout the blastocyst stage in marsupials, because of preventing early implantation, constitutes a primary constraint on the evolution of extended gestation periods in this lineage. To my knowledge, this is the first comparative single-cell-based implantation study. Our data allows us to revisit the evolutionary origin of eutherian implantation and reconsider its homolog or evolutionary predecessor in marsupials.

9. *Cell-cell communication at the fetal-maternal interface displays predicted integration, disambiguation, and signaling continuity patterns.*

Throughout my thesis, I have focused on the importance of signaling in female reproductive biology. Cell-cell communication is critical to any tissue function, and inferring signaling from single-cell transcriptomics has become common. This approach is central to studying phenotypes by their relational status, particularly the fetal-maternal interface and implantation.

We drew upon classical conceptual frameworks to consider co-evolutionary signaling dynamics between the mother and the fetus (Haig, 1996). We also introduced more recent theoretical work on homeostatic signaling between different cell-type pairs and cell-type circuits (Adler et al., 2023; Pavlicev & Wagner, 2024) contributing to tissue function. In the last two chapters of my thesis, I modified these broad predictions and applied them to cell-cell communication inferred from single-cell data.

We first tested the prediction of increased functional integration of fetal and maternal cell types during placentation. We found that the decidua and invasive trophoblast display higher levels of non-autocrine signaling. We interpreted the higher proportion of allocrine ligands (with the receptor unexpressed) and allocrine receptors (ligand unexpressed) as a sign of more dependence upon other cell types and, hence, more integration into the tissue.

Along with changes in life history traits, parent-offspring conflict may have driven some aspects of placental evolution. This theory predicts that fetal-maternal signaling will evolve toward a maternal-only or fetal-only expression to avoid singling redundancy and maintain maternal

physiology, resulting in disambiguation. Some of the fetal-only or maternal-only ligands correspond to maternal-specific and placental-specific genes. The latter derives from placental-specific promoters, endogenous retroviruses, and the evolution of large gene families through the emergence of paralogs (Rawn & Cross, 2008). We found a pattern of disambiguation and origin-specific expression of gene families such as Wnt, prolactin, chorionic somatomammotropin, steroids, and immunomodulatory signals. Conversely, signals involved in homeostatic maintenance, such as vascular endothelial growth or platelet-derived growth factors, displayed a more bilateral expression.

Our research has revealed two consistent patterns, integration, and disambiguation, across all the species we studied, including the opossum. This observation further connects us to the last prediction we tested on our data, presented in the last chapter of my thesis. We anticipated that a post-implantation interface in eutherians requires some signaling continuity with pre-implantation to ensure a long gestation period. Specifically, we asked, when the trophoblast replaces the maternal epithelium during invasive placentation, does it have to replace the lost epithelial signals? By comparing the gene expression of the maternal epithelium to the cell types present at the fetal-maternal interface, we found that trophoblast populations, particularly invasive subtypes, displayed the highest similarity scores. That said, the cytotrophoblast of the opossum also produces a secretome that is highly similar to luminal epithelium. We interpret this as evidence that the similarity of trophoblast and epithelial cells was an enabling factor in the evolution of placental implantation, alongside maternal innovations, such as the origin of the decidua, to bring about the tolerance of the implantation event.

This new approach to analyzing single-cell transcriptomic data, which applies theoretical predictions to cell-cell communication data, further opens the possibilities of comparative single-cell studies. As computational tools to infer cell-cell communication from single-cell data increase rapidly, novel ways of interpreting this type of data are valuable and applicable to the fetal-maternal interface and other systems.

Future Directions

The main aspects of female reproductive biology still need investigation in most eutherian species, particularly within the early branching Xenarthra and Afrotheria taxons. More data from a broader range of species is essential to fully understand the diversity of the mechanisms involved in maternal recognition of pregnancy and in extending gestational progesterone. Only this can provide a better understanding of ancestral patterns, too. Specifically, elucidating the mechanism for maternal recognition of pregnancy in species from the orders Pilosa and Cingulata would be of great interest. Furthermore, a better understanding of the role of progesterone during marsupial gestation could improve our understanding of the evolution of eutherian pregnancy from the luteal phase.

My results indicate that while the role of Wnt signaling in embryo receptivity is an eutherian innovation, other gene expression hallmarks are already present in marsupials and only later acquired a specific implantation function. The study of implantation in more eutherian species is essential to gain better insight into the conservation of pathways and what is genuinely novel in eutherian implantation. Lastly, more comparative studies on marsupial gestation are necessary to understand if eutherian implantation evolved from post-attachment cell-cell contact, as proposed, and to harmonize this view with the lack of broad similarities at the single-cell level in the opossum.

Placental diversity and variation are central tenets of my thesis. However, our current understanding of mammalian pregnancy is primarily based on mouse and human. Having found significant differences in placental patterns even among more closely related species, such as mouse and guinea pig, further underscores the importance of studying non-model species. The work I have done in my thesis is an additional step in that direction. Further research on additional species is

necessary to further discuss cell type homology at the fetal-maternal interface. We have proposed that an endocrine decidua was a derived evolutionary acquisition in placental mammals. One way to confirm this model is to assess decidualization at the single-cell level in other Afrotherian or Xenarthran species. Using approximately homologous gestational stages was relevant to my study. I have studied the fetal-maternal interface once fully formed before luteolysis or luteal-placental shift occurs. While I consider this critical when researching the fetal-maternal unit of new species, these stages also limit our ability to learn the process of early trophoblast invasion and decidual response in detail. The latter and any insights into trophoblast developmental trajectories in non-model species would require analyzing earlier time points.

Cross-species single-cell analysis is a newly emerging field whose methods still need to be fully optimized. Cell-cell communication inferred from single-cell data is relatively straightforward. However, a meaningful interpretation is not. Some of the limitations to correctly interpreting this type of data came from expected sources: the lack of manually curated repositories of ligands and receptors, the constraint of only including human orthologs and losing paralog information, the random assignment of only one transcript as the given ortholog and, of course, the presence of false inferred ligand-receptor interactions can only be confirmed by experimental validation and functional evidence. Other challenges of providing a meaningful interpretation of this data type were less anticipated. We have slightly changed fundamental theories of broad predictions and applied them to cell-cell communication from single-cell data. Still, some of the signaling dynamics found may not be exclusively specific to eutherian pregnancy, as it was also present in the marsupial outgroup. More research is thus necessary to determine those cell-cell interactions underlying invasiveness and placental types in eutherians.

For a long time, metaphors have permeated and informed cultural and scientific representations of pregnancy (Filippini, 2020). Also, the speculations and theories on the evolution of the fetal-maternal interface have invoked different metaphors (Pijnenborg et al., 2008). Taken cautiously, metaphors can inspire novel ways of approaching complex data on the fetal-maternal interface. For instance, more work is needed to correctly assess the criteria for recognizing a signature of maternal-fetal conflict in cell-cell communication data. More broadly, new signaling sources emerge during evolutionary transitions and developmental novelties that engage with preexisting cell types and communication circuits. Investigating changes in signaling potential from different cell types during these transitions, with some cell populations changing substantially and others preserving the same role, can open up new research avenues for evolutionary questions.

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