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Table of contents

List of figures

List of abbreviations

Cp = Ceruloplasmin

CHO = Chinese Hamster Ovary

CTB = Cytotrophoblast

CYB561A3 = Cytochrome b ascorbate-dependent protein 3

DFO = Deferoxamine

DMEM = Dulbecco`s Modified Eagle Medium

DMT1 = Divalent Metal Transporter 1

DPBS = Dulbecco`s Phosphate Buffered Saline

DTPA = Diethylenetriaminepentaacetic acid

DcytB = Duodenal cytochrome B

FAC = Ferric ammonium citrate

FBS = Fetal Bovine Serum

FECs = Fetal endothelial cells

FPN1 = Ferroportin 1

Ft = Ferritin

Ft-H = Ferritin heavy chain

Ft-L = Ferritin light chain

GAPDH = Glyceraldehyde-3 phosphate dehydrogenase

GCL = Glutamate cysteine ligase

GCLC = Glutamate cysteine ligase catalytic subunit

GCLM = Glutamate cysteine ligase modifier subunit

GF-AAS = Graphite Furnace Atomic Absorption Spectroscopy

γ-GC = γ-glutamylcysteine

GPX = Glutathione peroxidase

GR = Glutathione reductase

GS= Glutathione synthetase

GSH = Reduced glutathione

GSSG = Oxidized glutathione

HCP1 = Heme-Carrier protein 1

Heph = Hephaestin

HIF = Hypoxia inducible factor

HFE = Human hemochromatosis protein

HRP = Horseradish Peroxidase

hTCs = Human trophoblast cells

IDA = Iron deficiency anemia

IRE = Iron Responsive Element

i.v. = Intra-venous

KD = Knockdown

LIP = Labile iron pool

LOD = Limit of detection

Nrf2 = Nuclear factor-E2-related factor 2

NTBI = Non-transferrin bound iron

PKC = Protein kinase C

RBCT = Red blood cell transfusion

rHuEPO = Recombinant human erytrophoietin

RIPA = Radioimmunoprecipitation assay

ROS = Reactive Oxygen Species

RT = Room temperature

SD = Standard deviation

siRNA = Small interfering RNA

shRNA = Short hairpin RNA

STB = Syncytiotrophoblast

STEAP3 = Six trans-membrane epithelial antigen of the prostate 3

TBI = Transferrin-bound iron

TBP = TATA-binding protein

TBST = Tris-buffered saline with Tween 20

Tf = Transferrin

TFR1 = Transferrin Receptor 1

UBC = Ubiquitin C

UTR = Untranslated region

WHO = World Health Organisation

ZIP = ZRT/IRT like protein

1. Introduction

1.1 Iron in a biological context

Iron is a trace element that plays a pivotal role for all biological domains of life [1]. This relevance might by a consequence of its high natural abundance, since iron is the most ubiquitous member of the transition metals and with a percentage of roughly 5% the fourth most common element in the Earth`s crust [2], [3].

In addition, iron possesses remarkable chemical properties for electron transfer reactions, because it can accommodate eight different redox states ranging from -2 to +6 [2]. However, the biologically relevant forms of iron are the ferrous (Fe^{2+}) and ferric (Fe^{3+}) state [4]. To a minor extent also iron species with a higher oxidation number naturally occur during transition states of some enzymatically catalysed reactions [5].

In cells, iron can be incorporated into three classes of proteins: Iron-sulfur cluster proteins, hemeproteins or non-heme/non-iron-sulfur proteins [6]. As a co-factor, iron is involved in many cellular and physiological processes including:

- Oxygen transport and storage: In heme-containing metalloproteins an iron atom is coordinated in the center of the porphyrin ring, which allows binding of oxygen to the prosthetic group [7]. In red blood cells, the tetrameric hemeprotein hemoglobin is responsible for oxygen delivery from the respiratory organs to the remnant tissues of the body [8]. In contrast, the monomeric hemeprotein myoglobin, located in muscle cells, is functionally adapted to store oxygen [9].
- Energy metabolism: Oxidative phosphorylation in the mitochondria is a key metabolic process that generates energy in form of ATP by transfer of electrons from an acceptor to a terminal donor [10]. Several proteins that are involved in these transfer reactions exert their function via iron-sulfur clusters [2]. At this point it is further noteworthy, that the ability to gain energy solely from oxidation or reduction of iron is still preserved in some organisms [11].
- DNA synthesis: It has been known for many decades that iron is involved in the process of DNA synthesis [12]. By this time we know that several members of the DNA replication as well as repair machinery require iron as a co-factor to ensure genome stability [13]. One of the best characterized representatives is the enzyme ribonucleotide reductase that uses iron to generate tyrosyl radicals for the reduction of ribonucleotides to deoxyribonucleotides [14].

Despite its necessity for functionality of life, unbalanced iron levels pose a threat to all cells by the generation of reactive oxygen species (ROS) [15]. Most of the intracellular iron is bound to proteins, while only a small proportion occurs as "free" iron in the redox-reactive, transitory labile iron pool (LIP) [16]. The LIP fuels the generation of ROS via the Haber-Weiss reaction, which combines reduction of ferric iron and the Fenton reaction [17].Hereinafter, this oxidative stress has detrimental consequences for the cells due to ROS-mediated DNA damage and lipid peroxidation [15]. In the last decade, an iron-dependent form of non-apoptotic cell death has been described, termed ferroptosis [18]. In the current model ferroptosis is the result of lipid hydroperoxide accumulation as an aftereffect of perturbances in the biosynthesis of the major cellular antioxidant glutathione [19]. While the biological reason for ferroptosis is still unknown, its involvement in several illnesses, including acute kidney failure and liver injury, has been established [20], [21]. Furthermore, treatment with ferroptosis-inducing agents has been suggested as a novel approach for cancer treatment, since cancer cells seem to be especially susceptible to this kind of cell death [18], [22].

1.2 Iron metabolism

The metabolism of iron is a tightly regulated system that has to precisely coordinate the consumption and distribution of iron to ensure the wellbeing of the organism (Figure 1) [23].

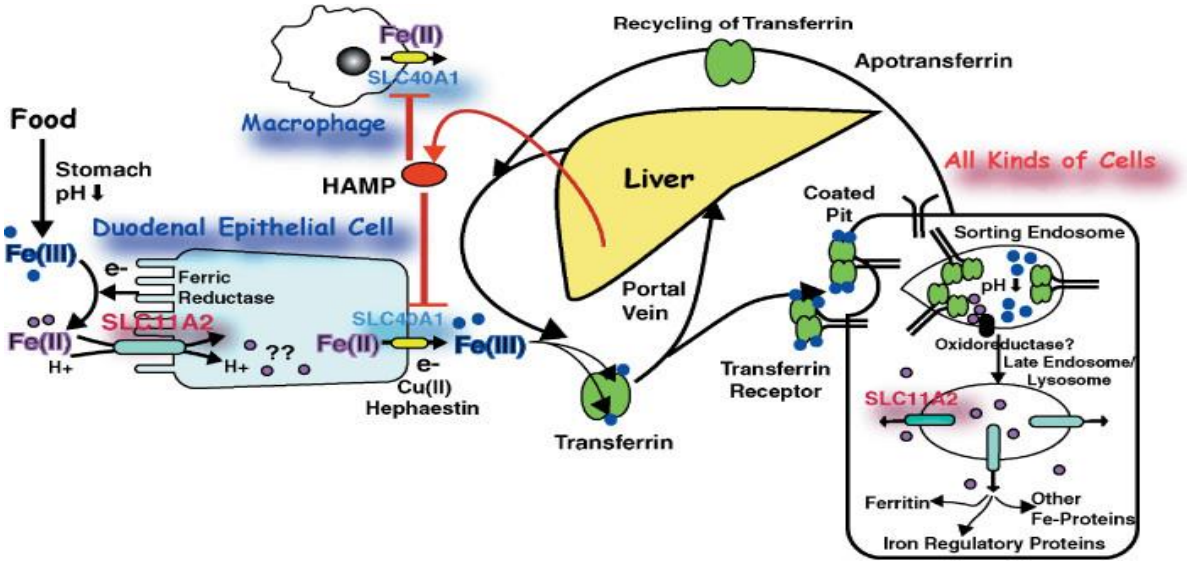


Figure 1. Summary of iron metabolism. Ferrous iron is taken up via DMT1 (*SLC11A2*) by the enterocytes of the duodenum. Upon its basolateral efflux by FPN1 (*SLC40A1*) and subsequent re-oxidation to ferric iron, it is bound to transferrin. Transferrin-bound iron is taken up by endocytosis of the transferrin receptor 1-transferrin complex. From the endosome iron is transported to the cytosol via DMT1 and can be either stored in proteins or effluxed by FPN1. The main regulator of systemic iron levels is the liver produced peptide hormone hepcidin (*HAMP*), which controls the levels of FPN1 [23].

Iron is mainly taken up as dietary iron, which can be further classified into heme (Fe^{2+}) and non-heme (Fe^{3+}) iron, by the enterocytes of the duodenum [24], [25]. Red meat is the main source for heme iron, in form of hemo-and myoglobin, while non-heme iron is abundantly provided by white meat and vegetables in form of ferric salts [26]. Although the exact mechanism of heme iron absorption is unknown, it is hypothesized that it occurs by receptor-mediated endocytosis of heme-carrier protein 1 (HCP1) [27]. Prior to uptake, non-heme iron has to be reduced to ferrous iron by either reducing agents like ascorbic acid or by duodenal cytochrome b (DcytB), a ferrire-ductase located at the apical site of differentiated enterocytes [28], [29]. Ferrous iron is then fur-ther transported across the membrane by divalent metal transporter 1 (DMT1), a proton-coupled symporter encoded by the *SLC11A2* gene [30].

In the cytoplasm iron undergoes two distinct fates, either storage, or export at the basolateral side of the enterocyte [31]. The main iron storage protein is ferritin (Ft), a globular multi-subunit complex consisting of 24 heavy (Ft-H) and light chains (Ft-L) that can store up to 4500 iron at-oms [32]. Before storage, ferrous iron is converted to ferric iron by the oxygenase activity of Ft-H [33].

To reach the circulation iron has to be transported across the basolateral membrane by the only known iron exporter, Ferroportin 1 (FPN1) [34]. FPN1 is a 62 kDa multipass transmembrane protein encoded by the *SLC40A1* gene [35]. Mutations of FPN1 may cause the autosomal domi-nant disease hemochromatosis type IV resulting in iron overload as a consequence of resistance to hepcidin regulation [36].

At the basal side, ferrous iron has to be re-oxidized by the ferroxidases hephaestin (Heph) or ceruloplasmin (Cp) to bind in its ferric form to transferrin (Tf) [37]. Transferrin, a 80 kDa glyco-protein mainly synthesized in the liver, is the most important iron transporter in the blood stream [38]. The two-lobed transferrin molecule has two binding sites for ferric iron that require car-bonate or an equivalent anion for coordination [39]. However, these binding sites are not fully occupied all the time, which results in three different transferrin states: apo-transferrin (no iron bound), mono-ferric-transferrin (one iron atom bound) and holo-transferrin (two iron atoms bound) [40]. In the plasma, the most prevalent state is apo, followed by monoferric, while only a small proportion of the total transferrin is in the diferric state, thereby creating a pool to stem the threat of sudden iron overload [41].

The cellular uptake of transferrin-bound iron (TBI) is mainly mediated by the transferrin receptor 1 (TFR1) (Figure 2) [42].

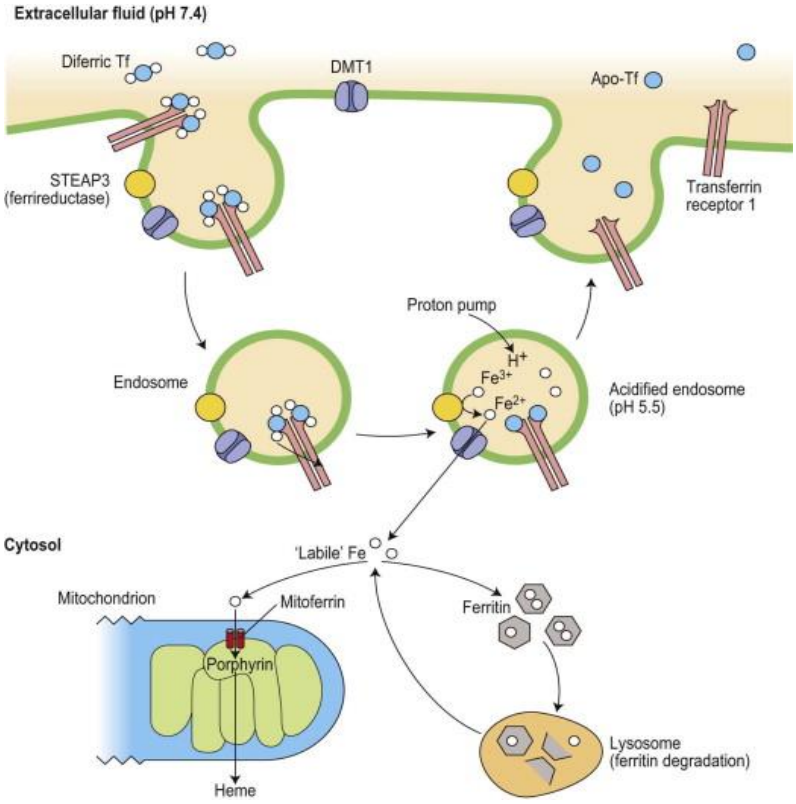


Figure 2. Cellular iron uptake via Transferrin Receptor 1. Graph from [42].

TFR1, encoded by the *TFRC* gene, is a homo-dimeric transmembrane glycoprotein consisting of two disulfide-bond linked monomers [43], [44]. Each monomer consists of three globular domains: an apical, an interconnecting helical and a protease-like domain [45]. Structure determination has revealed that the helical and protease-like domains are the docking sites for iron-loaded transferrin molecules [46]. Conformational changes of transferrin upon iron binding enhance its association with the receptor [46], [47]. As a consequence, diferric iron has by far the highest association constant of all three Tf species, whereas the apo-protein has virtually no chance of stable interaction with TFR1 under physiological conditions [41], [48].

Once iron-bound transferrin associates with TFR1 the resulting complex is internalized via clathrin-mediated endocytosis [49]. During endosome maturation, the vesicle becomes acidified, which leads to several protonation reactions culminating in the release of iron from the transferrin molecule [46], [50]. The resulting apo-transferrin remains tightly bound to the receptor in this acidic environment and is recycled back into the bloodstream, where it dissociates at the neutral pH [51].

In the endosome ferric iron is reduced to ferrous iron by ferrireductase six trans-membrane epithelial antigen of the prostate 3 (STEAP3) [52]. Fe^{2+} is then transported into the cytosol via DMT1 [53].

While iron in the blood is mainly bound to transferrin or other iron binding proteins like ferritin and hemoglobin, also a special form of free iron exists, termed non-transferrin bound iron (NTBI)[54]. Its major forms have been described to be ferric citrate and ferric acetate [54]. NTBI uptake is especially important in the liver, where it is transported into the cell by a member of the ZRT/IRT-like family, ZIP14, upon reduction to ferrous iron [55].

1.3 The threat of iron deficiency and iron overload during pregnancy and perinatal period

According to the World Health Organisation (WHO) iron deficiency is the most common form of micro-nutritional deficiency worldwide [56]. Per definition, iron deficiency is characterized by a lower total body iron amount (below the normal serum iron concentrations for an average adult, which ranges from 10-30 µM). Low serum-ferritin levels (<30 µg/L) and diminished transferrin-saturation (≥ 16%) are indicative for this condition. In addition to malnutrition, the major causes for iron deficiency include chronic bleeding (e.g., menstruation or frequent blood donations), malabsorption syndromes (e.g., as a consequence of a gastrectomy) or parasitic infections [57].

The demographic groups that are most prone to suffer from iron deficiency are young women and children. Especially pregnant women are at high risk due to elevated iron demands during pregnancy [57]. During the first trimester of pregnancy iron requirements decrease because blood loss through menstruation is lacking. However, from the second trimester onwards, iron demands continuously increase in the third trimester up to 10 times the amount of a non-pregnant woman to supply fetal erythropoiesis and proper development of placenta and fetus. Importantly, dietary uptake is insufficient to provide these high iron amounts and therefore women should begin pregnancy with filled iron stores to by-pass this discrepancy [58].

Up to 80% of a term new-born's total body iron is acquired during the third trimester. Consequently, pre-term infants (<37 week of gestation) are almost always affected by iron deficiency, in which iron levels are too low to maintain normal physiological functions. Untreated iron deficiency, may lead to iron deficiency anemia (IDA) that is defined in infants as a hemoglobin concentration of less than 11 g/dL. IDA has severe after-effects for the fetus that manifest in impaired neuro-development [59].

The easiest and cheapest way to treat IDA is oral supplementation with iron salts like ferrous sulphate (recommended daily iron doses: 100-200 mg for adults; 3-6 mg/kg for infants). However, oral iron supplements often have adverse effects on the gastro-intestinal system including nausea, vomiting and diarrhea and insufficient absorption is common [57]. If oral iron treatment is not tolerated or ineffective, parenteral iron supply is an alternative approach. The intra-venous

(i.v.) administration of iron compounds (most widely used are iron-carbohydrate complexes) has several advantages to oral supplementation including faster and higher rise of hemoglobin concentration and restoration of body iron stores [60]. Although modern i.v. iron compounds are in general considered as very safe, parenteral dextran iron products have been associated with anaphylactic reactions [60], [61]. A second way of parenteral iron administration is red blood cell transfusion (RBCT). RBCT has been an anemia treatment for a long time, but is nowadays mainly used if chronic bleeding occurs or hemoglobin levels fall under a certain threshold. A major concern on blood cell transfusion is the transmission of infectious diseases [62]. Furthermore, RBCT is especially dangerous in the treatment of premature infants, since it has been associated with the development of parenteral nutrition associated liver disease [63].

Another alternative to treat IDA, is the administration of recombinant human erythropoietin (rHuEPO). Combination of rHuEPO and parenteral iron has been shown to be very effective in the treatment of IDA in pregnancy [64]. However, several side-effects have been described for rHuEPO treatment including hypertension, thrombosis, hyperkalaemia and anaphylactic reactions [65]. Furthermore, rHuEPO treatment is associated with a non-significant increased risk of retinopathy of prematurity [66].

Since iron homeostasis is tightly regulated, iron overload can have as detrimental consequences as iron deficiency for proper development in the fetal and neonatal period [67]. Iron overload diseases in infants have been associated with fetal growth retardation, lactic acidosis and liver hemosiderosis [68]. Although iron overload has also been suggested to cause neurodevelopmental disorders in premature infants, a recent study found no such connection [69]. In addition to several genetic disorders (e.g. hemochromatosis), iron overload may also have iatrogenic causes like unnecessary, prophylactic iron supplementation [67].

In summary, iron supplementation during pregnancy can improve the iron status of the mother and protect the infant from IDA to some extent. It is still highly debatable whether this iron supplementation should occur routinely or selectively, although neither can prevent IDA completely during pregnancy [70]. It has to be considered that shortage as well as excess of iron are harmful for both the developing fetus and infant and therefore (prophylactic) iron supplementation is highly controversial in the treatment of iron deficiency [71]. Especially, in preterm infants oral iron supplements should be avoided due to the infant`s potential vulnerability to the iron-mediated oxidative stress [67].

1.4 The placenta

The human placenta starts to develop in the pre-implantation stage (approximately 4-5 days after conception). During the transition from the morula to the blastocyst, the outer most cell layer differentiates into the trophoblast that surrounds the inner cell mass (embryoblast) and blastocoel. While the inner cell mass will give rise to the embryo, umbilical cord and extraembryonic mesoderm, the trophoblast will constitute the main part of the extraembryonic tissues (placenta and fetal membranes) [72].

On day 6-7 after conception, the blastocyst hatches from its surrounding glycoprotein layer (zona pellucida) and attaches to the uterine epithelium that has developed into an embryo-receptive tissue, called decidua [72], [73]. At this time, the mononuclear trophoblast cells overlying the inner cell mass (polar trophoblast) differentiate into an oligonucleated syncytium, termed syncytiotrophoblast (STB). The invasive properties of the STB allow the conceptus to penetrate the uterine epithelium and to embed in the decidual stroma. Eventually the early embryo is surrounded completely with the STB, which expands by means of an underlying mononucleated layer of trophoblastic stem cells named cytotrophoblast (CTB) [72].

From day 8 after conception ahead, fluid-filled spaces occur within the STB and fuse into larger lacunae. As soon as the lacunae have formed, the placenta can be categorized into three distinct components: an embryo-facing early chorionic plate, the lacunar system with the trabeculae (remaining syncytiotrophoblastic masses between lacunae) and the primitive basal plate that is in contact with the uterine epithelium [72].

As the syncytiotrophoblast penetrates deeper into the decidual stroma, it erodes the dilated maternal capillaries, named sinusoids [72], [74]. Thereby maternal blood eventually enters the lacunar system. Upon completion of implantation, around 12 days post conception, extraembryonic mesoderm cells migrate on top of the inner CTB surface, thereby forming the outer fetal membrane, termed chorion. At the same time, cytotrophoblasts from the chorionic plate travel to the basal plate, differentiate into extravillous cytotrophoblasts and invade the decidual stroma. A subgroup of these cells, the endovascular trophoblasts, then destroys the walls of maternal spiral arteries and replaces it with trophoblasts, thereby founding the basis for a hemochorial uteroplacental circulation system [72],[75].

From day 13 post-conception onward, the trabeculae begin to form projections, termed primary villi, which may already contain a cytotrophoblastic core (Figure 3A). Afterwards, the extraembryonic mesodermal cells of the chorionic plate penetrate into the primary villi and give them a mesenchymal core, thereby transforming them into secondary villi (Figure 3B). Mesodermal

cells differentiate into first placental blood cells and blood vessels, which marks the transition to tertiary villi (Figure 3C) [72].

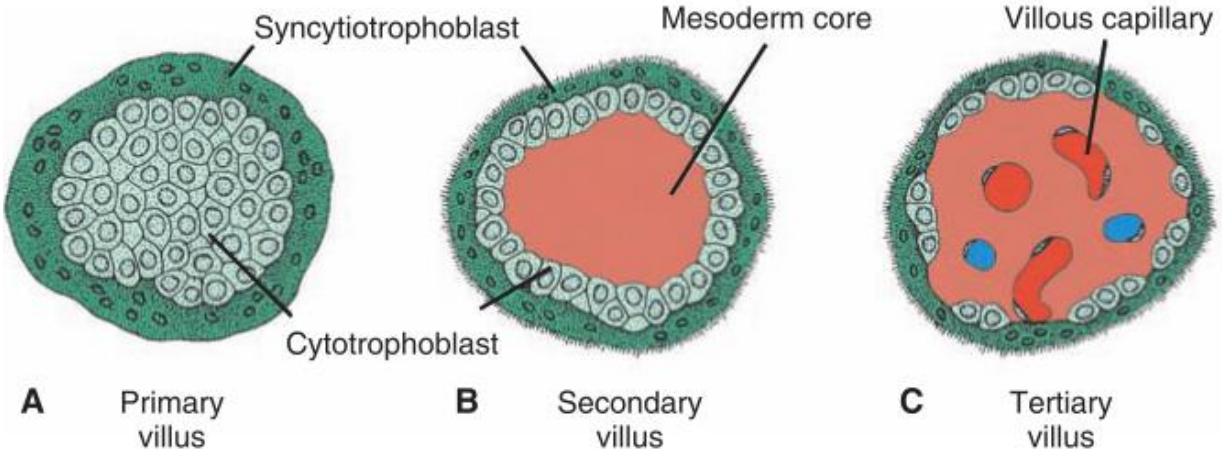


Figure 3. Stages of chorionic villi formation. Picture from [75].

The villous trees arise from the chorionic plate by a villous stem that branches off many freely floating villi in the intervillous space (formerly known as lacunae), where they are in direct contact with the maternal blood. The architecture of the villous tree increases the surface area for feto-maternal exchange [72].

While the basal plate represents the maternal surface of the placenta, its fetal surface is represented by the chorionic plate. The chorionic plate is covered by the amnion, the second fetal membrane. Inserted into the chorionic plate lies the umbilical cord, which is the blood conduit connecting fetus and placenta (Figure 4) [72], [76]. The fully developed umbilical cord is established during the 12th week of gestation and consists of two arteries and one vein, which are embedded in a gelatinous substance called Wharton`s jelly. The umbilical vein transports nutrient-rich, oxygenated blood from the placenta to the fetal heart, while oxygen-repleted blood is transported back to the placenta by the umbilical arteries [76].

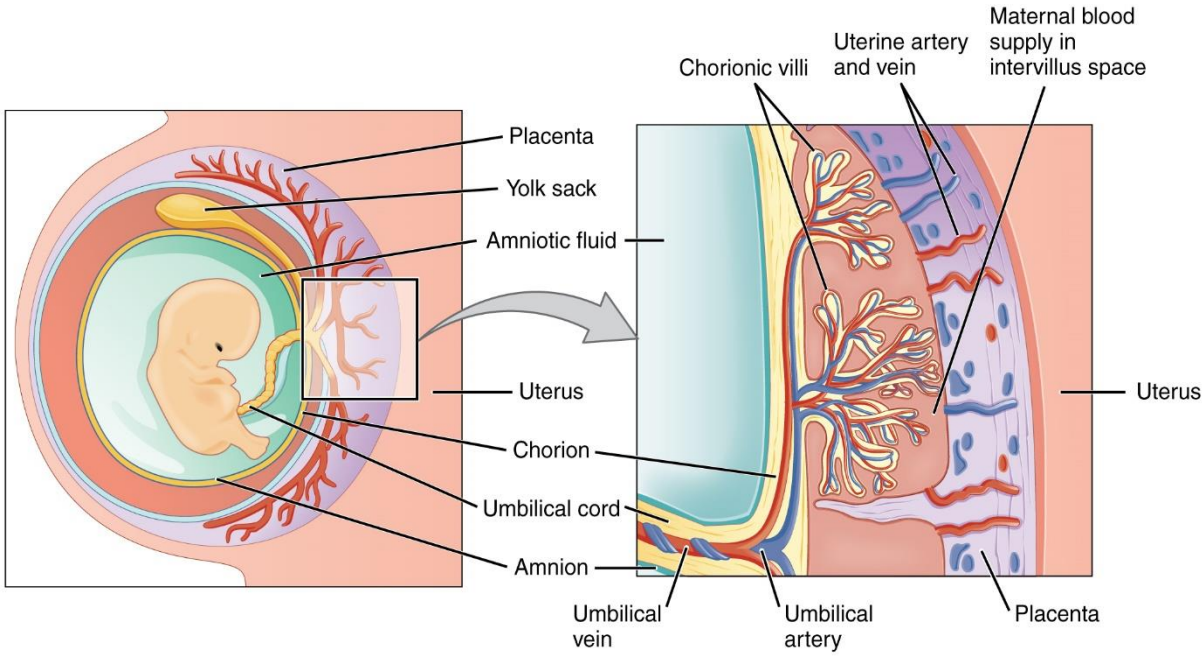


Figure 4. Structure of the human placenta. In the uterus the embryo resides within the amniotic sac, which is constituted of the outer chorion and inner amnion. Due to the hemichorial character of the human placenta, the chorionic villi are in direct contact with the maternal blood. The umbilical vein transports oxygen-rich blood to the fetus, while de-oxygenated blood is transported back to the placenta via the umbilical arteries [72], [76].
Picture obtained from: https://courses.lumenlearning.com/ap2/chapter/embryonic-development/

Anatomically the placenta changes considerably during the process of gestation. In the first trimester, the human placental barrier, which separates maternal and fetal circulations, is a multilayered structure consisting of fetal endothelial cells (FECs), basal membrane, CTB and STB. During progression of pregnancy the CTB becomes discontinuous, which facilitates exchange processes between mother and fetus [77], [78].

1.5 Human placental iron metabolism

Although mammalian iron metabolism is in general very well characterized, the knowledge of placental iron metabolism is far more fragmentary (Figure 5). Even less is known about the specifics of the human placental iron metabolism, because the main insights on the molecular mechanisms of iron transport across the placenta come from animal models [79]. Since the human placenta possesses distinct molecular features that are not found in other animals, particular care is required for making assumptions on the human system from these studies [80]. In addition to these observations, most of our current understanding of the iron metabolism in the human placenta is based on either localization studies or *in vitro* cell culture experiments [81], [82].

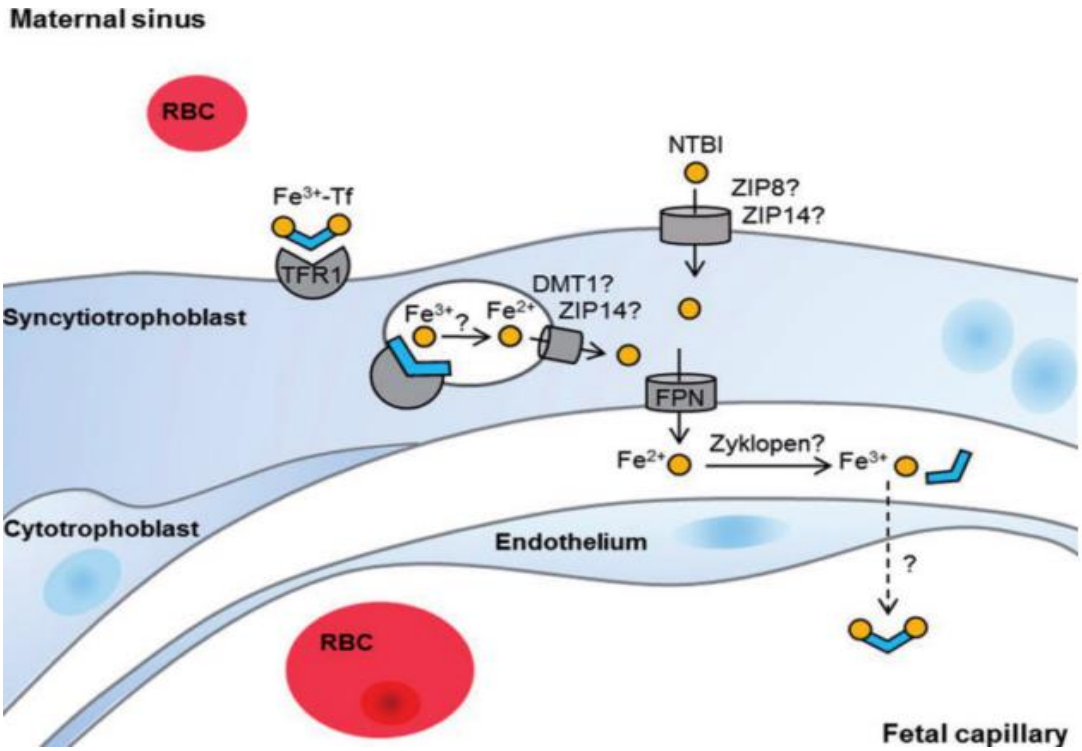


Figure 5. Current knowledge of the human placental iron metabolism. Transferrin-bound iron uptake occurs at the syncytiotrophoblast via TFR1. In the endosome iron is reduced and transported into the cytosol by an unknown mechanism that could involve DMT1 or ZIP14. Additionally, the placenta may also take up non-transferrin bound iron (NTBI) by ZIP14 or ZIP8. From the syncytiotrophoblast iron may be exported by FPN1 and oxidized to the ferric form (possibly by zyklopen). It has been suggested that Fe^{3+} then binds to fetal transferrin, before it enters the fetal endothelium [79].

The most important iron source in placental iron metabolism is maternal transferrin-iron, which is taken up by TFR1 [79]. TFR1 is highly abundant in the placenta and predominantly localized on the apical maternal-facing side of the syncytiotrophoblast [81]. Both in mice and humans, placental TFR1 expression depends on the maternal iron status, suggesting a conserved mechanism that ensures the fulfilment of fetal iron demands via regulation of TFR1 levels [82], [83]. Its pivotal role in placental iron metabolism has been indicated by studies in *Tfr* $^{-/-}$ knockout mice that were anemic from embryonic day (E) 10.5 onwards and died before E 12.5 [84].

Although it is highly suggestive that TBI-uptake at the STB occurs via a classical transferrin cycle (see Figure 2), the exact molecular mechanism has not been elucidated [79]. The endosomal reduction of ferric iron upon its release from Tf may be exerted by STEAP3 or its homolog STEAP4, which are both abundantly expressed in the human placenta [52], [111].

Another conundrum is the identification of the endosomal transporter responsible for iron export into the cytosol [79]. Studies in *Dmt1* knockout mice have demonstrated that the protein is dis-

pensable for materno-fetal iron transfer, since the viable offspring was not iron iron-deficient at birth. However, shortly after birth these pups developed severe anemia and died, as soon as DMT1 would have been required for intestinal non-heme iron absorption [86].
It has been suggested that ZIP14 may serve as an additional endosomal iron transporter in the placenta [79]. Placental expression of ZIP14 on the mRNA level was reported in humans [87]. Although in one publication ZIP14 expression was also claimed for the mouse placenta, no further investigations in this regard were made [88]. However, like DMT1, ZIP14 seems to be dispensable for placental iron transport, since *Slc39a14* knockout mice gave birth to phenotypically unsuspicious offspring with normal iron levels [89].

In addition to TBI, the placenta may also take up other iron sources, namely NTBI and heme iron [79]. The most promising candidate for NTBI uptake in the placenta is the zinc and iron transporter ZIP8 (*SLC39A8*), a close homolog of ZIP14. Hypomorphic *Slc39a8 (neo/neo)* mice that exhibited decreased ZIP8 levels in embryo, fetus, placenta, yolk sac and several neonatal tissues were severely anemic and died between gestational day 18.5 and 48h postnatally. Moreover, this study suggested an indispensable role of ZIP8 for iron uptake in embryonic and fetal hematopoietic organs as well as in general embryonic development [90]. The upregulation and enhanced cell surface localization of ZIP8 in response to iron overload was demonstrated in rat hepatoma cells and may be a general mechanism for NTBI uptake to cope with excess iron [91]. In the human placenta, ZIP8 is highly expressed and localized at the apical side of the STB (Ellinger, unpublished 2017). Suppression of endogenous ZIP8 in a human choriocarcinoma cell line resulted in a 40% reduction of NTBI uptake. For the first time, these observations strongly indicate a role for ZIP8 in placental iron metabolism, although it concomitantly raises the demand for a so far undetermined ferrireductase [91].
Recent studies further suggest a mechanism for utilization of heme iron sources to support fetal iron demands, since placental heme transporters were upregulated under suboptimal iron conditions [92].

Tracer studies with radioactively labelled iron in primary cytotrophoblasts demonstrated the incorporation of transferrin-derived iron into ferritin [93]. Although placental ferritin expression has been characterized for a long time, its localisation within the organ has been highly controversial [79], [94]. The most consistent ferritin expression and thereby potential iron storage capacity function has been described for fetal villous stromal cells and placental macrophages (Hofbauer cells) [79], [95].

Furthermore, maternal serum-ferritin has been suggested as an alternative iron source during pregnancy as ferritin receptor levels increased at the microvilli membrane upon iron depletion [96]. In accordance, radioactive tracer studies in animal models showed the materno-fetal transport of ferritin-derived iron [97].

Due to its localisation at the basal membrane of the syncytiotrophoblast, FPN1 is the most likely iron exporter in the placenta [81]. FPN1 is highly expressed in the placenta, where it is localized on the basal fetal-facing membrane of the STB [81]. Its importance for maternal-fetal iron transport has been demonstrated in mice that had decreased placental FPN1 expression due to a deletion of the IRE (Iron Responsive Element) in the 5` UTR (Untranslated Region). As a consequence, neonatal pups suffered from iron deficiency and severe anemia [98].

FPN1 is regulated by the small peptide-hormone hepcidin, which binds to the transporter and triggers its degradation [31].The role of both, maternal and fetal hepcidin in placental iron metabolism has not yet been fully elucidated. During pregnancy, maternal hepcidin levels constantly decrease reaching a minimum in the third trimester. By keeping high levels of FPN1, this phenomenon has most probably the purpose to sustain increased fetal iron demands by allowing enhanced dietary iron uptake and mobilization of iron stores. Fetal-derived hepcidin may on the other hand directly regulate the iron amount that reaches the fetal circulation via control of syncytiotrophoblastic FPN1 levels [99]. Hepcidin-mediated regulation of placental iron transport is further indicated by studies in mouse models that hyper-express the peptide hormone. Constitutive hepcidin expression results in impaired materno-fetal iron transport, which caused severe anemic neonates [100]. Consequently, a role in placental iron metabolism has also been suspected for HFE (Human hemochromatosis protein), the regulator of hepcidin synthesis. Studies in *Hfe* knockout mice showed that maternal Hfe levels were critical for iron transport across the placenta. Under adequate iron supply conditions, heterozygous offspring from knockout dams accumulated more iron in comparison to the ones from wild-type dams. This observation was attributed to the inability of the knockout mice to down-regulate placental TFR1 and FPN1, despite sufficient iron levels. Furthermore, also fetal Hfe levels seem to be involved in placental iron transport, since pups of knockout mice lost their ability to control iron acquisition during high maternal dietary intake [101]. In the human placenta, HFE is highly expressed. Localization studies in the human choriocarcinoma cell line BeWo as well as in primary trophoblasts showed HFE to be associated with TFR1 in endosomes. This raised the possibility for a direct regulative function of HFE in TBI iron uptake [102].

The fate of iron after its release from the STB is very poorly described, but it was presumed that it enters the fetal tissues upon binding to transferrin. If this is the case, after FPN1-mediated export, iron must be oxidized to its ferric form before binding to fetal transferrin [81].
The ferroxidase Ceruloplasmin (Cp) is highly expressed in the placenta, where it is localized in the intervillous space, STB and fetal capillaries. A protective role for Cp by preventing excessive ROS formation, rather than a direct involvement in iron transport was suggested [103]. The dispensability of Cp for placental iron trafficking was further demonstrated in *Cp* $^{-/-}$ mice that showed a normal phenotype at birth [104]. Expression of a close Cp homolog, hephaestin, was described in BeWo cells [105]. Radioisotope studies in mice carrying a hephaestin mutation, suffered from anemia and showed long-term defects in placental iron transfer to the developing fetus [106]. A third putative placental ferroxidase, zyklopen, was originally identified in human choriocarcinoma cells. Zyklopen shows strong sequence similarity with Cp and hephaestin. Immunostaining in mice demonstrated its absence in liver and intestines, but high expression in placenta and yolk sac. Its role in iron efflux is further suggested by structural analysis, as zyklopen is a membrane-bound protein that possesses the proper topology for a direct interaction with FPN1 [107].

The expression patterns of iron transporters amongst several tissues change during pregnancy in accordance to the fetal iron demands. Placental levels of ZIP8 and FPN1 increase with gestational age, whereas maternal liver hepcidin and HFE levels decrease as pregnancy progresses. Furthermore, hepatic TFR1 and duodenal DMT1 expression increases during pregnancy to sustain elevated iron requirements. Interestingly, it has been reported that expression of TFR1 is unaffected by gestational age (samples from 24-40 week of gestation) in the placentas of iron-sufficient non-anemic women [90], [99], [108], [109].

1.6 *In vitro* models for human placental transport

Most of our current knowledge about transplacental iron transport is derived from *in vitro* models that can be categorized into three main classes: *ex vivo* placental perfusion models, tissue preparation models and cell culture models. Although the first two categories possess the closest similarity to the *in vivo* situation, they are effortful and technically challenging, which urges the development of sophisticated cell culture systems [77].
Placental cells that have been used in transport studies can either be primary trophoblasts directly isolated from the placenta or immortalized cell lines [110], [111]. While the primary cells have the advantage that they can be obtained from different stages of gestation as well as from patho-

logical pregnancies, they are short-lived, prone to contamination with other cell types and they do not proliferate [77], [112] .

One of the best established placental cell lines, is the human choriocarcinoma cell line BeWo [113]. Morphologically, they resemble primary trophoblasts and express similar marker proteins [114]. BeWo cells usually mimic the cytotrophoblast, but can be stimulated to fuse forming a syncytiotrophoblastic phenotype [115]; this in contrast to the BeWo-derived cell line Jeg-3 that shows spontaneous syncytialization [114].

The original b24 clone does not form confluent monolayers under standard culture conditions [116]. Therefore, the b30 subclone was generated, which forms confluent monolayers on its own. These cells can be grown on semi-permeable transwell membranes to form a polarized barrier that can be employed in transport studies [117]. Amongst many other applications, this system has already been used to gain insights into the placental transport of iron [111].

1.7 Aim of the study

The aim of this study was to elucidate iron uptake into human placenta cells. Silencing of the transporters TFR1 (*TFRC*), DMT1 (*SLC11A2*) and ZIP8 (*SLC39A8*) via small interfering (si)RNA mediated gene knockdown was employed to perform functional studies on iron accumulation in the human choriocarcinoma cell line BeWo as well as in human primary trophoblast cells obtained from healthy term placentas. In particular, the present study aimed to:

1) Determine the role of TFR1, DMT1 and ZIP8 in placental iron uptake

2) Determine gene expression patterns of FPN1, FTH1, ZIP14, STEAP3, CYB561A3, GCLM, GPX1 and GR upon silencing of TFR1, DMT1 and ZIP8.

3) Determine whether there are functional differences on iron metabolism in the cancer cell line BeWo and primary trophoblastic cells

2. Material and Methods

Detailed protocols of the stated methods are provided in the appendix.

2.1 Cell culture

The b24 clone of the human choriocarcinoma cell line BeWo [113] was a kind gift of Dr. Isabella Ellinger (Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna). HeLa cells were from American Type Culture Collection (ATCC, #CCL-2).

Cells were maintained at 37°C in 95% humidity/5% CO_2 in Dulbecco`s Modified Eagle Medium (DMEM) high glucose (Gibco) supplemented with 10% FBS (Fetal Bovine Serum) (PAN Tech) and 1% Glutamax (Gibco). Cells were subcultured once they reached 60-90% confluency and detached from culture dishes with 0.25% trypsin/EDTA (Gibco). Cell number was obtained with CASY cell counter and analyser (CASY ® Model TTC 45/60/150, Innovatis, Germany).

Primary human trophoblast cells (hTCs) isolated from term placenta by Elisabeth Straka and Sebastian Granitzer as described previously [118], were cultured in Keratinocyte-SFM (serum free medium) (Gibco) supplemented with 10% FBS, 1% Glutamax. Patients gave written informed consent and the ethics committee of the Medical University of Vienna (EC number 833) approved the study.

2.2 siRNA mediated gene knockdown

For standard knockdown experiments, BeWo cells were seeded at a density of 5 x 10^4 or 1 x 10^5 cells/well in 12-well or 6-well plates (CytoOne) respectively. After 8 to 10 hours, when approximately 25% confluent, cells were transiently transfected with non-targeting siRNA (si control) and siRNA targeting *TFRC*, *SLC11A2* and *SLC39A8* (Dharmacon GE) (Supplementary Table 1). The lipid based forward transfection of BeWo and Hela cells with Lipofectamine RNAiMax (Life Technologies) was performed as previously described [119]. In case of BeWo cells, only ¼ of the original transfection reagent amount was employed. Afterwards cells were cultivated until harvest at 63 hours post transfection.

hTCs were seeded at a density of 1 x 10^6 cells in 6-well plates and concomitantly transfected (reverse knockdown) [118] using Lipofectamine 2000 (Invitrogen) according to the manufacturer`s instructions. Cells were cultivated until harvest at 72 hours post transfection.

2.3 Cell harvest

In general, cells were handled on ice and centrifuged at 200 x g. Medium was collected and pooled for further analysis. Cells were washed with cold DPBS (Lonza), detached with trypsin/EDTA for 5-7 min at 37°C, then resuspended in 1 ml DPBS and subjected to CASY measurements (1:200 in CASYton Buffer). Afterwards, triplicates were pooled and washed twice with DPBS. Half of the cells was used for AAS measurements, the other half for knockdown verification.

After pooling, the cells grown in 6-well plates were incubated for 3 min in 1 ml DPBS (50 µM diethylenetriaminepentaacetic acid (DTPA) (Sigma)), washed once with DPBS and equally split for AAS measurements, protein extraction and RNA isolation.

hTCs were washed with DBPS and incubated with 1 ml DPBS (50 µM DTPA) for 1 min. Afterwards cells were washed once with DPBS, detached using cell scraper (CytoOne), washed with DPBS and also equally split for AAS measurements, protein extraction and RNA isolation.

2.4 Protein extraction and Immunoblotting

Cells were lysed for 20 min on ice in RIPA (Radioimmunoprecipitation assay) buffer containing (50 mM Tris, pH 7.6, 150 mM NaCl, 1% Triton, 0.1% SDS, and 0.5% sodium deoxycolate) supplemented with 2 mg/ml aprotinin, 0.3 mg/ml benzamidin chloride, 2 mg/ml leupeptin, and 10 mg/ml trypsin inhibitor (Sigma). Supernatants were collected upon centrifugation at 21382 x g for 20 min at 4°C and stored at -20°C.

Protein concentration was determined using the Bradford Assay (Biorad). 8-12 µg total protein were mixed with 4x Loading dye (200 mM Tris pH 6.8, 400 mM DTT (Biorad), 8% SDS (VWR), 0.4% Bromphenolblau (Merck), 40% Glycerol (Merck)), incubated for 10 min at 72°C, electrophoretically separated on 7.5-12.5% polyacrylamide gels, and transferred to a nitrocellulose membrane. Blots were blocked for 1 h in 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBST), followed by incubation in 5% BSA/TBST containing primary antibody over night at 4°C. Thereafter, blots were washed and incubated with corresponding secondary HRP (horseradish peroxidase)-conjugated antibodies. Enhanced chemiluminescence method (Pierce) was used to visualize signal. For a list of employed primary and secondary antibodies see Supplementary Table 2.

2.5 RNA isolation and cDNA synthesis and quantitative real-time PCR

Total RNA was isolated using TRI Reagent ® (Sigma) according to the manufacturer`s proto-

col. RNA was quantified using a Nanodrop-100 (Thermofisher Scientific). Up to 1000 ng purified RNA was reverse transcribed with Go-Script Reverse Transcription System (Promega) using random hexamer primers following the manufacturer`s instructions. The reverse transcription was performed in a thermocycler (Peqlab).

2.6 Quantitative real-time PCR

The cDNA was diluted 1:11 in nuclease-free water and 2 µl was used as a template in a 15 µl reaction. Gene expression was analysed using Taq Man Expression System (Applied Biosystems) in an Applied Biosystems StepOnePlus™ Real-Time PCR System according to the manufacturer`s instructions. Relative mRNA quantification was determined by the comparative Ct method using TATA-binding protein (TBP) and ubiquitin C (UBC) as reference genes. For a list of employed primers, see Supplementary Table 3.

2.7 Total iron measurements

Cell pellets were resuspended in 1 ml ddH_2O. Cell culture media were centrifuged for 3 min at 1800 x g to spin down debris. Then 1 ml of cell suspension or medium was mixed with 1.5 ml nitric acid (Rotipuran®Ultra, Roth) and digested in a microwave oven (MARS6, CEM, Germany) using the following program: 20 min to reach 200°C, hold 15 min at 200°C, cool down to 80°C. To further cool down the samples, they were kept at 4°C for 15 min. Then the opened vessels were placed under a laminar flow for at least 15 min to remove nitric acid waste gases. Finally, the vessels were rinsed with 1 ml ddH_2O to achieve a final water to acid ratio of approximately 2:1.

Iron content of sample solutions was analysed by graphite furnace atomic absorption spectroscopy (GF-AAS) (Z-8200 Polarized Zeeman AAS, Hitachi, Japan). A sample aliquot (20 µl) was introduced into the graphite tube, dried at 80-140°C, ashed at 750°C and atomized at 2400°C. If samples were out of the linear range (0-20 ppb iron), they were diluted in deionized water. The instrument`s limit of detection (LOD) was 0.3 µg/L. The mean iron level of reference material (Trace Elements Whole Blood L-2, Seronorm, LOT 1103129; LOT 1406264) was 256 µg/L (recovery: 77 ± 14%, N=3) and 263 µg/L (recovery 80 ± 15%, N=3) respectively. All samples were measured in duplicate by the working curve method (RSD < 15%).

2.8 FAC treatment

BeWo cells were cultured in 10 cm dishes (CytoOne). Knockdown for ZIP8 was performed as described above. After 38 h cells were washed with DPBS and either incubated with normal me-

dium (control) or DMEM containing 1% Glutamax, 100 µM ferric ammonium citrate (FAC) (Honeywell), and 100 µM ascorbic acid (Sigma) for 24 h until harvest.

2.9 Glutathione measurement

Intracellular glutathione levels were determined by the GSH/GSSG-Glo™ Assay (Promega). Cells were harvested and equally split for the analysis of total and oxidized glutathione. Cell pellets were lysed in 150 µl Total or Oxidized Glutathione Lysis Reagent for 10 min. Afterwards lysates were pipetted in triplicates into opaque flat-bottom 96 well-plates (Corning) and 50 µl Luciferin Generation Agent was added. After incubation for 30 min 100 µl Luciferin Detection Reagent were added into each well, followed by equilibration for 15 min. Luminescence was determined in a multiplate reader (Biotek).

2.10 Forskolin treatment

BeWo cells were grown until 50% confluency in 60 mm dishes (CytoOne). Cells were washed with DPBS and incubated with 20 µM Forskolin (Sigma) or normal medium (1% DMSO) as control. Cells were harvested after 24, 48 and 72h and subjected to gene expression analysis via quantitative PCR and immunoblotting.

2.11 Software and statistics

Data were processed using *Microsoft Excel 2013*. Statistical calculations and graphs were made with *GraphPad Prism6*. Bar graphs present mean values ± SD (standard deviation). Student`s paired t-test was used for mean value comparisons applying a critical significance level of $\alpha$=0.05. In case of less than three independent experiments, Student`s unpaired test was applied. *StepOne Software 2.3* was used for performance and analysis of qPCR. *Gene 5 1.10* software was used for analysis of the GSH/GSSG assay. *ImageJ* was used for densitometric evaluation of immunoblot signals.

3. Results

3.1 TFR1 knockdown

3.1.1 BeWo cells

Knockdown of TFR1 significantly reduced the number of BeWo cells (Figure 6A) and resulted in increased cellular iron levels in comparison to the control group (Figure 6B). In parallel, a non-significant trend for decreased iron concentrations in the cell culture media of TFR1 deficient cells was observed (Figure 6C). Immunoblotting confirmed the TFR1 knockdown (Figure 6D). Knockdown efficiency was quantified (82% ± 11%) by densitometry using *ImageJ* (Figure 6E). There was a strong positive correlation between knockdown efficiency and intracellular iron content upon TFR1 silencing (Figure 6F).

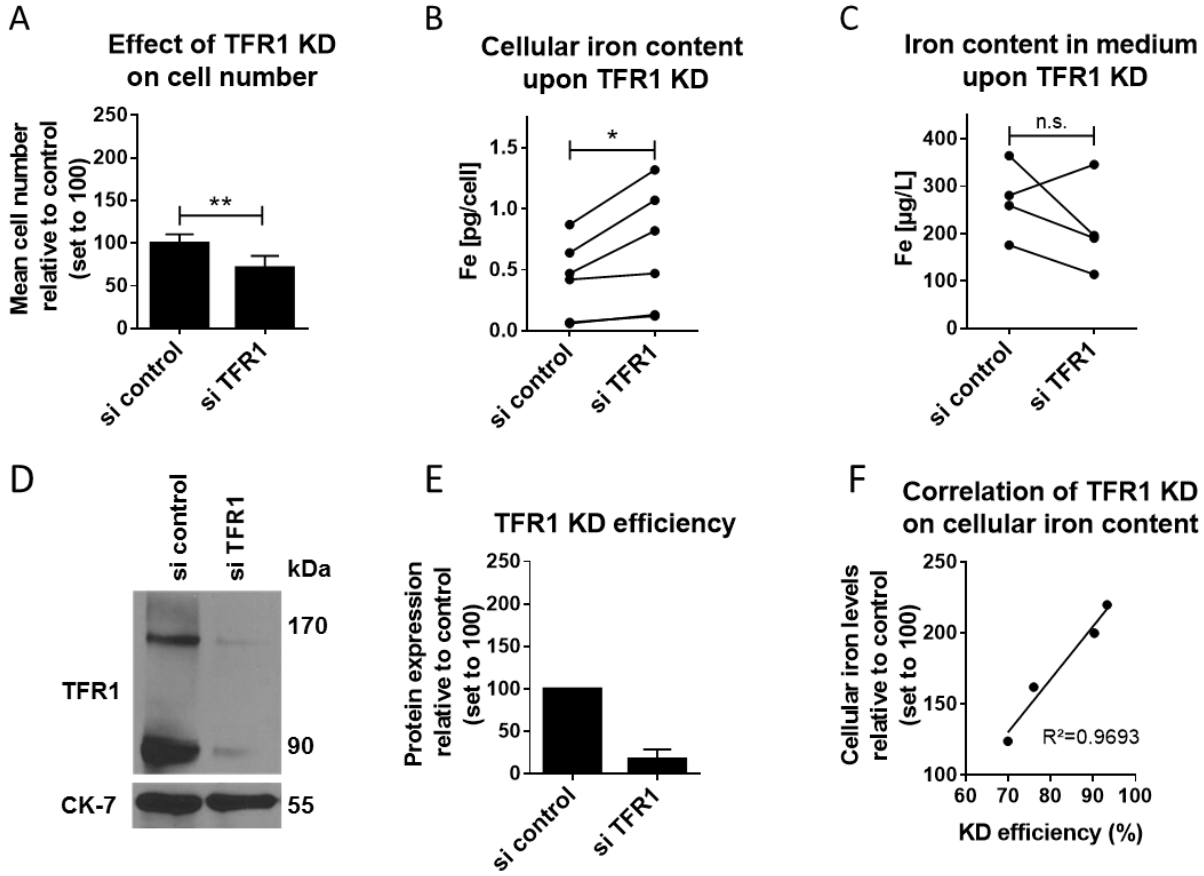
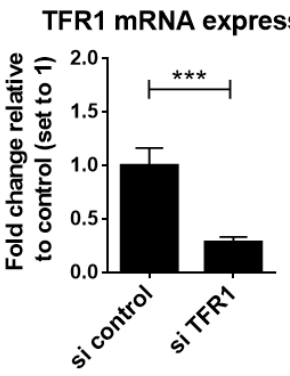


Figure 6. TFR1 knockdown in BeWo cells. A) Mean cell number was determined at the time of harvest. B) Cellular iron content normalized to cell number. C) Total iron content in the medium. D) TFR1 knockdown was confirmed by immunoblotting (one representative blot shown). E) The relative protein expression between control and knockdown was quantified by densitometry using *ImageJ*. F) Correlation between KD efficiency and cellular iron content. Data were obtained from at least 4 independent experiments. The bar graphs represent mean ± SD. n.s = $p>0.05$;*= $p<0.05$; **= $p<0.01$.

To determine whether silencing of TFR1 caused any alterations in the expression patterns of other proteins involved in iron metabolism, quantitative PCR was performed on RNA samples from TFR1 deficient cells (Figure 7A) and controls. While there was an up-regulation of FPN1 and FTH1 mRNA (Figure 7B, C) upon silencing of TFR1, no significant changes were observed for DMT1, ZIP8, ZIP14, as well as for the ferrireductases STEAP3 and CYB561A3 (Figure 7D-H). Analysis of ferrireductase STEAP4 is not shown, since its expression in BeWo cells was below the limit of detection. Furthermore, the involvement of the glutathione system was assayed and it was shown that upon TFR1 KD mRNA levels of glutamate cysteine ligase modifier subunit (GCLM) decreased, while glutathione peroxidase 1 (GPX1) increased and glutathione reductase (GR) showed only a slight tendency of reduction (Figure 7I-K).

Figure 7. Gene expression analysis upon TFR1 KD in BeWo cells. BeWo cells that showed decreased mRNA levels of TFR1 (A) upon siRNA treatment were analysed for gene expression levels of FPN1 (B), FTH1 (C), DMT1 (D), ZIP18 (E), ZIP14 (F), STEAP3 (G), CYB561A3 (H), GCLM (I), GPX1 (J) and GR (K). The data represent mean ± SD from two independent experiments. n.s = $p>0.05$;*= $p<0.05$; **= $p<0.01$, ***=$p<0.001$

A



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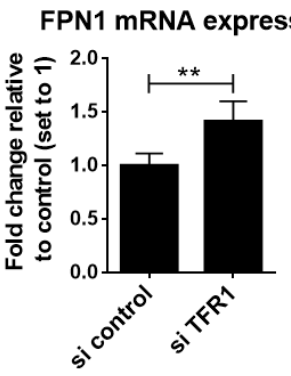

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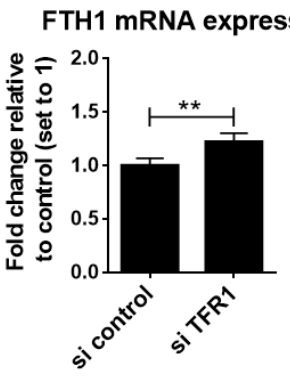

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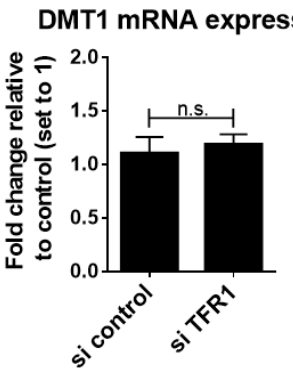

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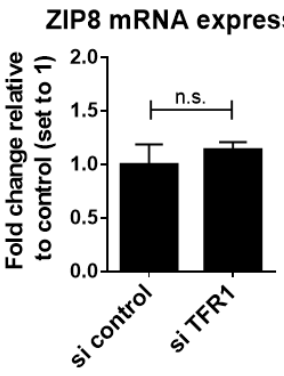

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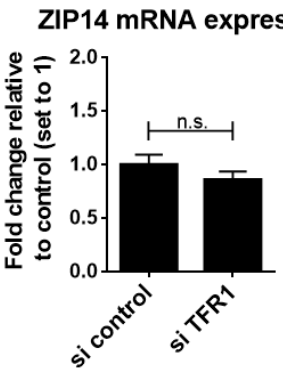
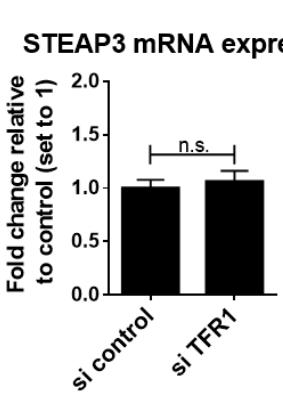
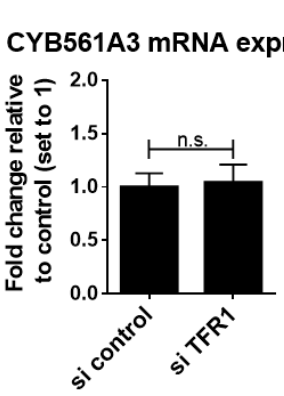


G



H



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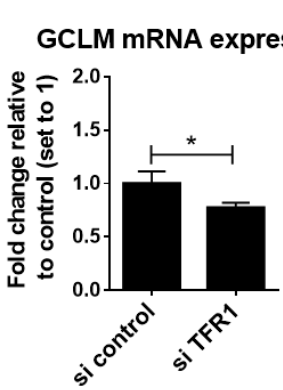

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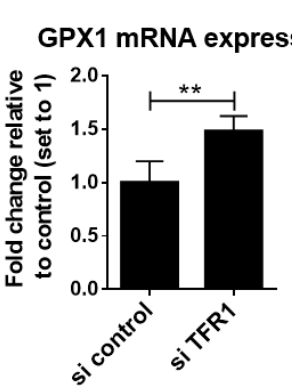

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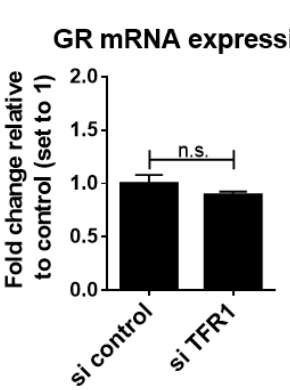

Cellular glutathione levels upon TFR1 knockdown were further analysed by the GSH/GSSG^{TM} Assay. While there was a tendency (p=0.0838) for increased total glutathione levels in TFR1 deficient cells (Figure 8A), the ratio of reduced (GSH) to oxidized (GSSG) glutathione was unchanged between control and knockdown treatment (Figure 8B).

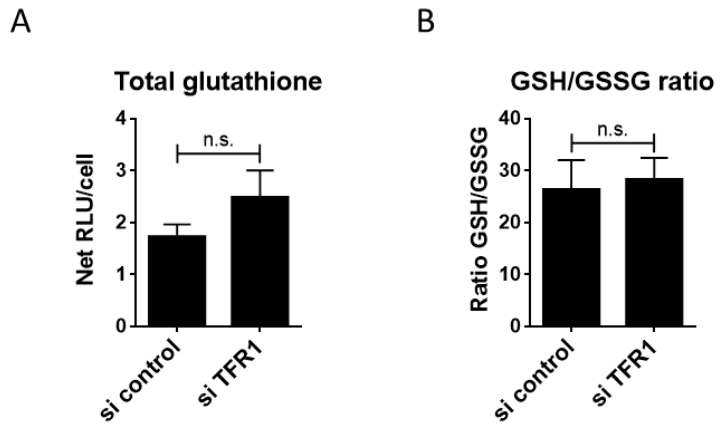

Figure 8. Glutathione in TFR1 deficient BeWo cells. A) Total glutathione levels are expressed as net Relative Light units (RLU) that were normalized to cell number. B) Ratio of reduced (GSH) to oxidized (GSSG) glutathione. The data represent mean ± SD from one independent experiment made in triplicates (each one of a different passage). n.s = p>0.05;

3.1.2 Human primary trophoblast cells

To determine whether the observations from BeWo cells correspond to the *in vivo* situation, functional studies were performed in primary human trophoblast cells, isolated from healthy term placenta. Because of the high variability in knockdown efficiency in the primary cells (trophoblast cells are hard to transfect), the results are given for each individual placenta.

In the first placenta, there was virtually no increase in cellular iron levels upon treatment with TFR1-targeted siRNA (Figure 9A), although cell culture medium iron levels were slightly decreased (Figure 9B). However, immunoblotting was unable to verify the knockdown (Figure 9C).

In the second case, TFR1 knockdown resulted in slightly elevated iron levels in the cells (Figure 9D) as well as in the medium (Figure 9E). Although the knockdown could not be verified by immunoblotting (data not shown), quantitative PCR revealed decreased TFR1 mRNA levels (65% of control) upon treatment with target-specific siRNA (Figure 9F).

In the third placenta, TFR1 silencing resulted in highly increased cellular iron levels (Figure 9.G), albeit iron levels in the medium were unchanged (Figure 9. H). In contrast to the previous experiments, knockdown could be clearly visualized by immunoblotting (Figure 11I).

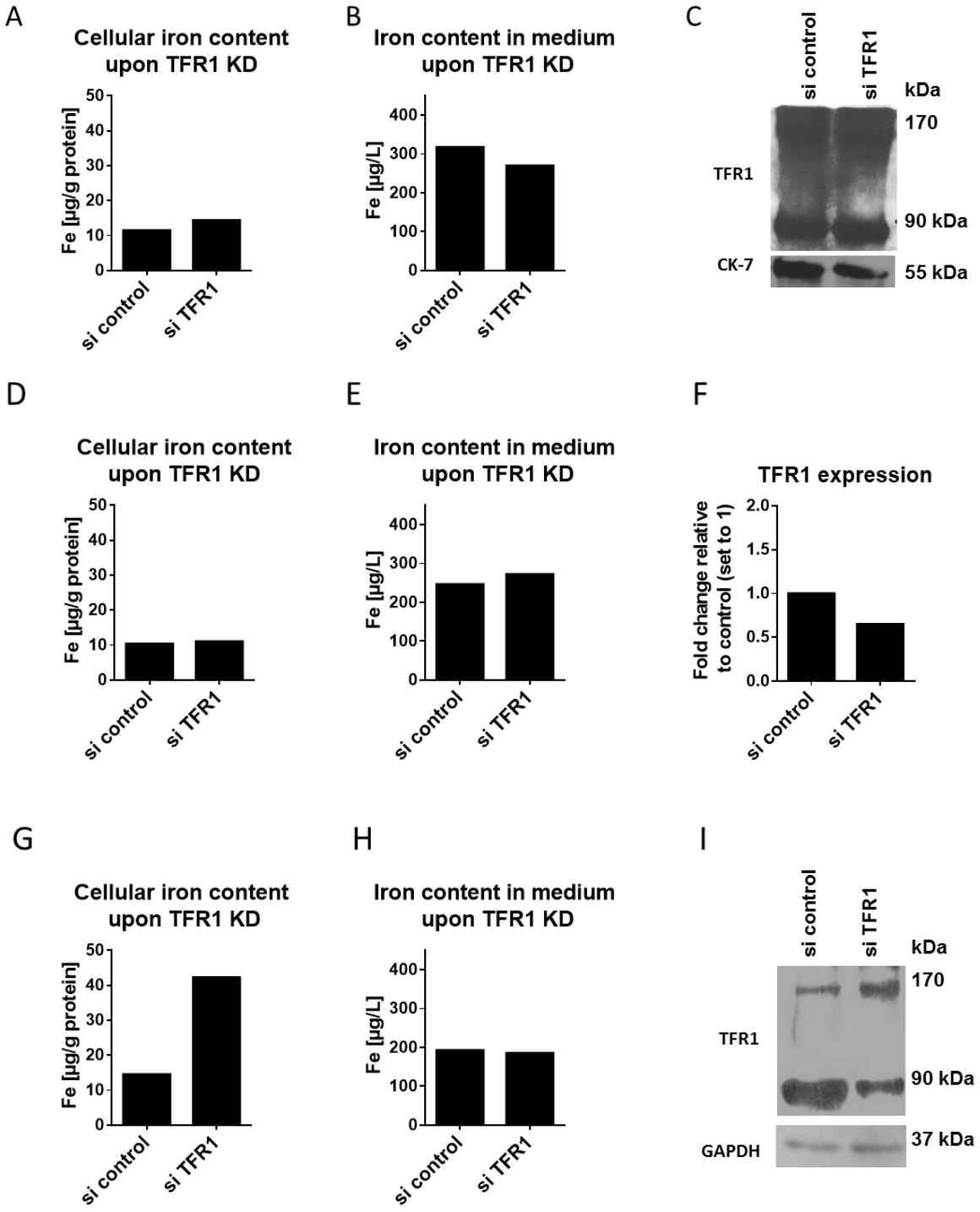

Figure 9. TFR1 knockdown in hTCs. Cellular iron content normalized to protein (A, D, G). Total iron content of the cell culture medium (B,E, H). Knockdown verification was either assayed by immunoblotting (C, I) or quantitative PCR (F). Results shown in A-C, D-F and G-I are from three different placentas.

3.2 DMT1 knockdown

3.2.1 BeWo cells

Knockdown of DMT1 had no apparent effect on cell number (Figure 10A). Upon DMT1 knockdown, intracellular iron levels were not uniformly increased or decreased (they were slightly elevated or decreased in comparison to the respective control) (Figure 10B). Iron content of cell culture medium did not differ between control and knockdown treatments (Figure 10C). Knockdown of DMT1 was highly efficient as demonstrated by immunoblotting (Figure 10D) and qPCR (Figure 11A).

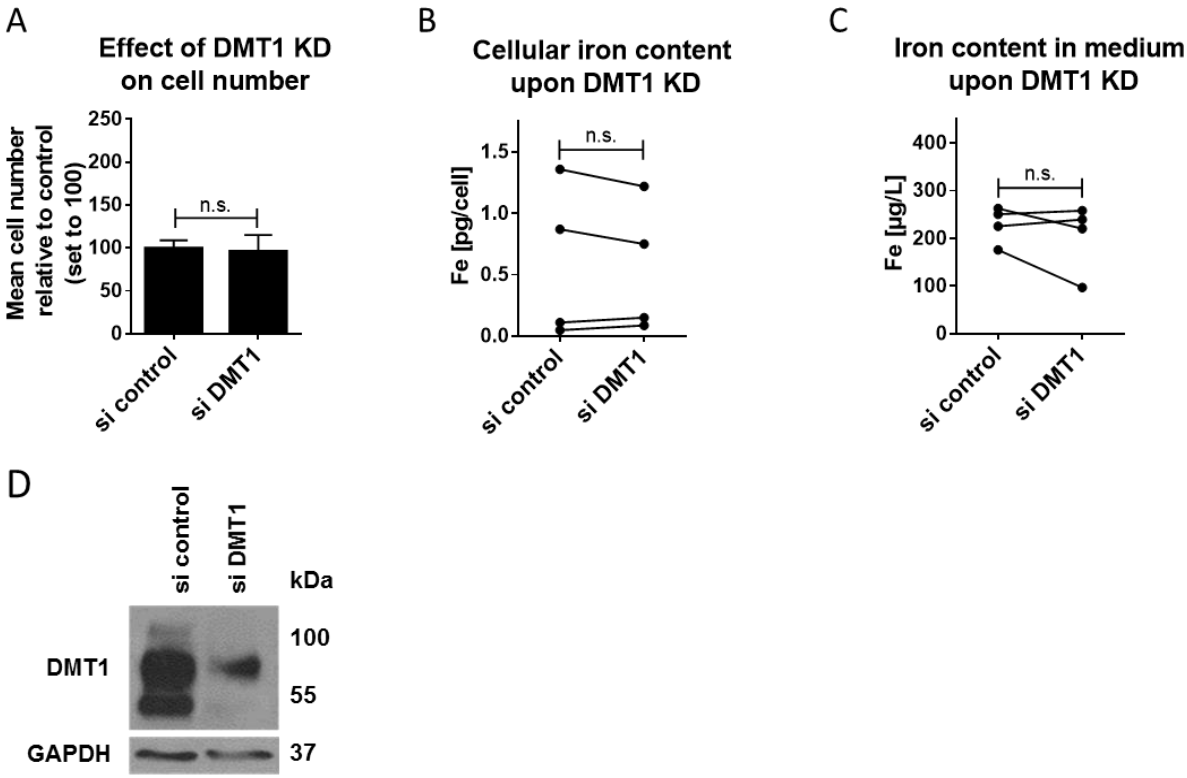

Figure 10. DMT1 knockdown in BeWo cells. A) Mean cell number was determined at the time of harvest. B) Cellular iron content normalized to cell number. C) Total iron content in the medium. D) DMT1 knockdown was confirmed by immunoblotting (one representative blot shown). Data were obtained from 4 independent experiments. The bar graphs show mean ± SD. n.s = p>0.05

In accordance with the results obtained from AAS measurements (higher iron content of cells upon DMT1 knockdown), the DMT1 deficient cells had increased levels of FTH1 mRNA (Figure 11B). No significant differences were observed for mRNA levels of STEAP3 and ZIP8 between control and DMT1 knockdown cells (Figure 11C, D). Interestingly, silencing of DMT1 resulted in decreased levels of ZIP14 mRNA (Figure 11E)

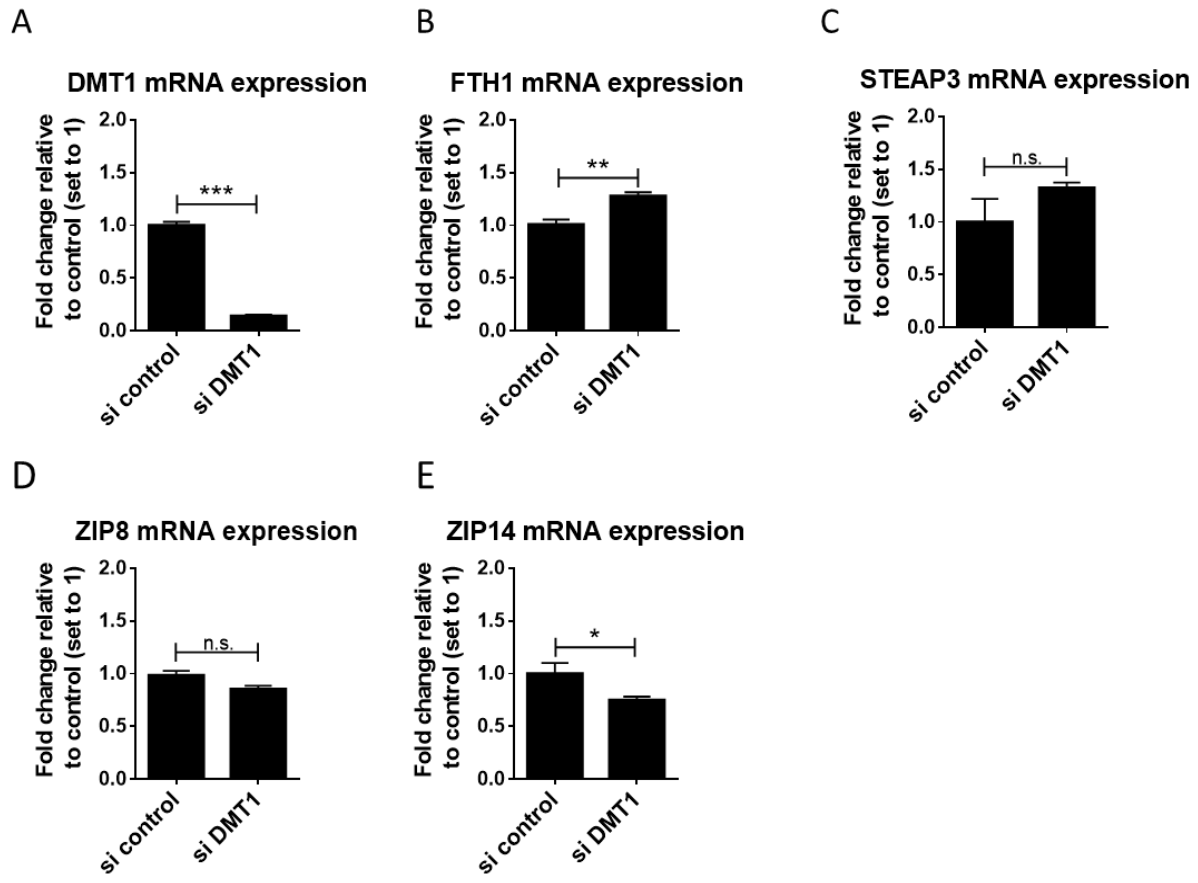

Figure 11. Gene expression analysis upon DMT1 KD. BeWo cells that showed decreased mRNA levels of DMT1 (A) upon siRNA treatment were analysed for gene expression levels of FTH1 (B), STEAP (C), ZIP8 (D) and ZIP14 (E). The data represent mean ± SD from two independent experiments. n.s = $p>0.05$;*= $p<0.05$; **= $p<0.01$;***=$p<0.001$

3.2.2 Human primary trophoblast cells

Upon DMT1 knockdown, primary trophoblasts brought similar results as BeWo cells. In the first case, cellular iron levels were decreased in DMT1 deficient cells (Figure 12A), whereas iron content in cell culture medium did not differ (Figure 14B). Knockdown efficiency (21%) was confirmed by quantitative PCR (Figure 12C).

In the second placenta, cellular iron levels were elevated in DMT1 knockdown cells (Figure 12D), whereas culture medium did not differ in iron content (Figure 12.E). However, in this case a very low knockdown efficiency (6%) was revealed by quantitative PCR (Figure 12F).

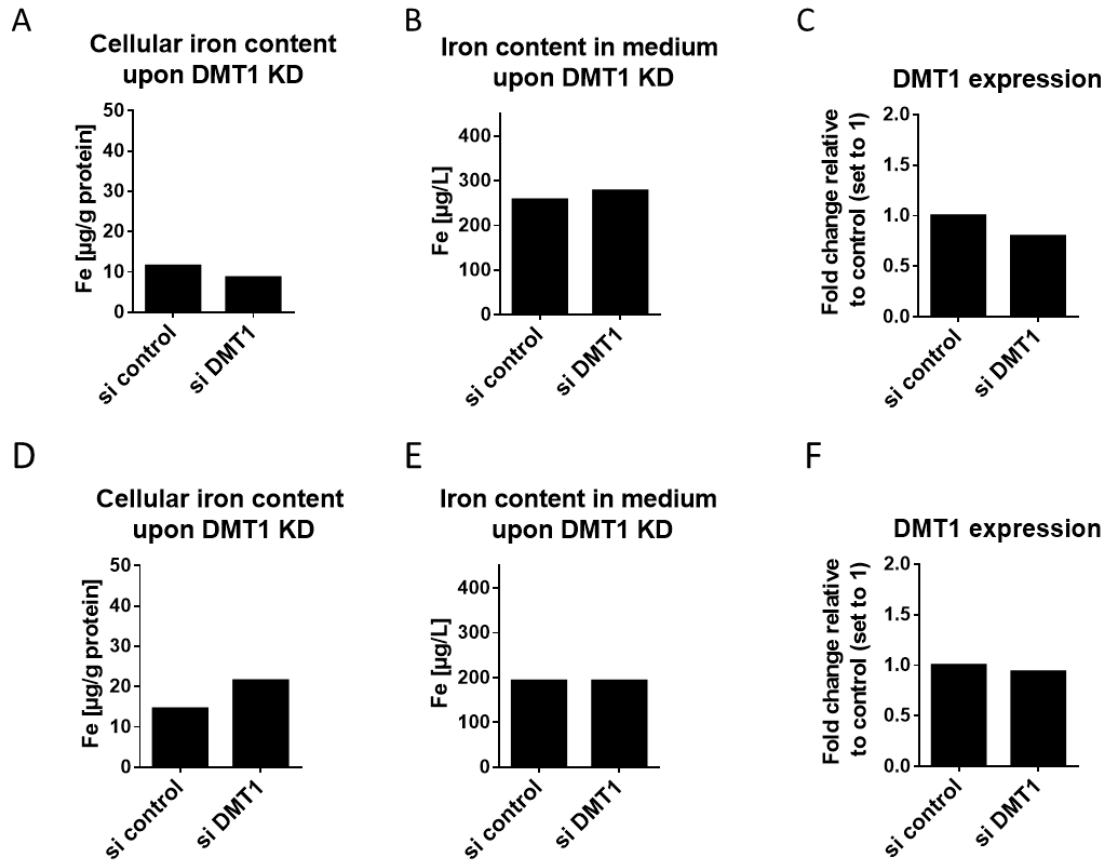

Figure 12. DMT1 knockdown in hTCs. Cellular iron content normalized to protein (A, D). Total iron content in cell culture medium (B,E). Knockdown verification (C, F). Results shown in A-C and D-F are from two different placenta.

3.3 ZIP8 knockdown

BeWo cell number was unaffected by silencing of ZIP8 (Figure 13A). Silencing of ZIP8 had also no effect on iron levels in cells (Figure 13B) or cell culture medium (Figure 13C). However, in the presence of ferric ammonium citrate as sole iron source, cells showed a tendency to accumulate less iron upon ZIP8 silencing (Figure 13D). The respective immunoblot provided a (rather weak) decrease in signal intensity at 130 kDa, the approximate protein size. In addition, it revealed the unspecific staining patterns of the employed ZIP8 antibody (Figure 13E). To demonstrate that this (130 kDa) region is the specific ZIP8 band, knockdown experiments were also performed in HeLa cells (Figure 13F).

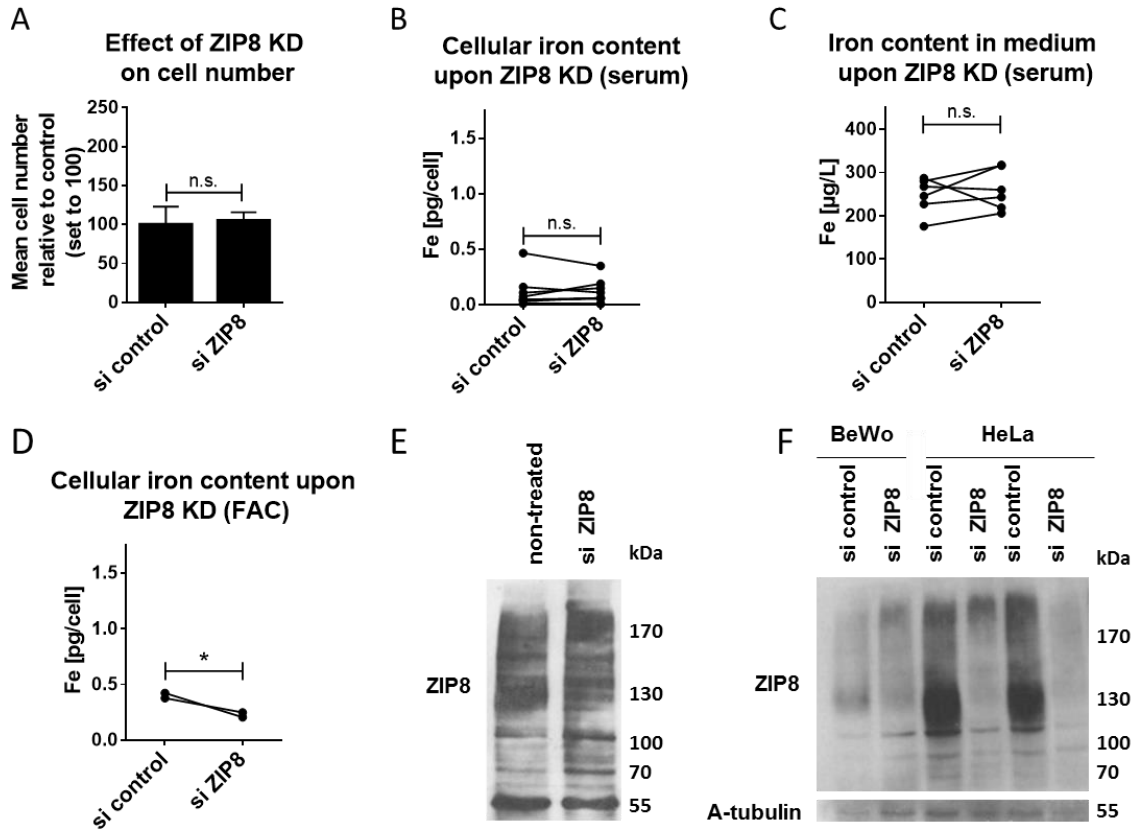

Figure 13. ZIP8 knockdown in BeWo cells. A) Mean cell number was determined at the time of harvest. B) Cellular iron content normalized to cell number. C) Total iron content in the medium. E) ZIP8 knockdown was analysed by immunoblotting (one representative blot shown). F) ZIP8 knockdown was performed in BeWo and HeLa cells to determine the specific band for the protein. Data were obtained from 4 independent experiments (FAC: one experiment in duplicate). The bar graphs show mean ± SD. n.s. = p>0.05

Knockdown of ZIP8 was verified also by quantitative PCR (Figure 14A). Furthermore, expression levels of DMT1, FTH1, STEAP3 and ZIP14 were analysed. These experiments revealed no changes in expression patterns between the controls and ZIP8 deficient cells for these proteins (Figure 14B-E).

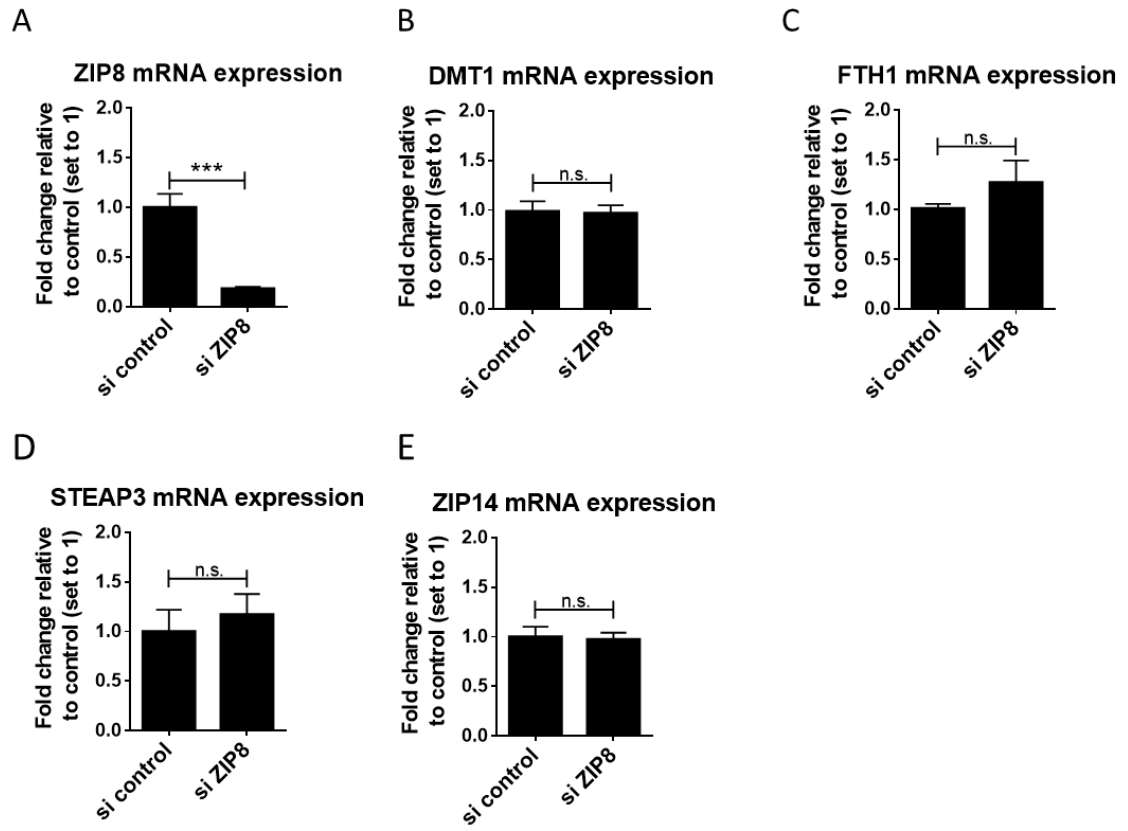

Figure 14. Gene expression analysis upon ZIP8 KD. BeWo cells that showed decreased mRNA levels of ZIP8 (A) upon siRNA treatment were analysed for gene expression levels of DMT1 (B), FTH1 (C), STEAP3 (D) and ZIP14 (E). The data represent mean ± SD from two independent experiments. n.s = p>0.05;***=p<0.001

3.4 Comparison of iron transporter expression between BeWo and hTCs

In order to determine whether BeWo cells express iron transporters in a similar way compared to primary trophoblast cells, gene expression analysis was performed by quantitative PCR. In case of DMT1, BeWo cells showed a tendency for higher expression than trophoblasts (Figure 15A). This finding was confirmed by immunoblotting (Figure 15B). In comparison to the BeWo cells, no band at 55 kDa was detected for DMT1 in any protein lysates from primary trophoblasts. On the other hand, primary trophoblasts showed a higher expression of ZIP8 (Figure 15C). Furthermore, the expression of TFR1 was also elevated in trophoblasts in comparison to the BeWo cells (Figure 15D). In general, the primary trophoblasts showed high individual variability in the expression of the target proteins.

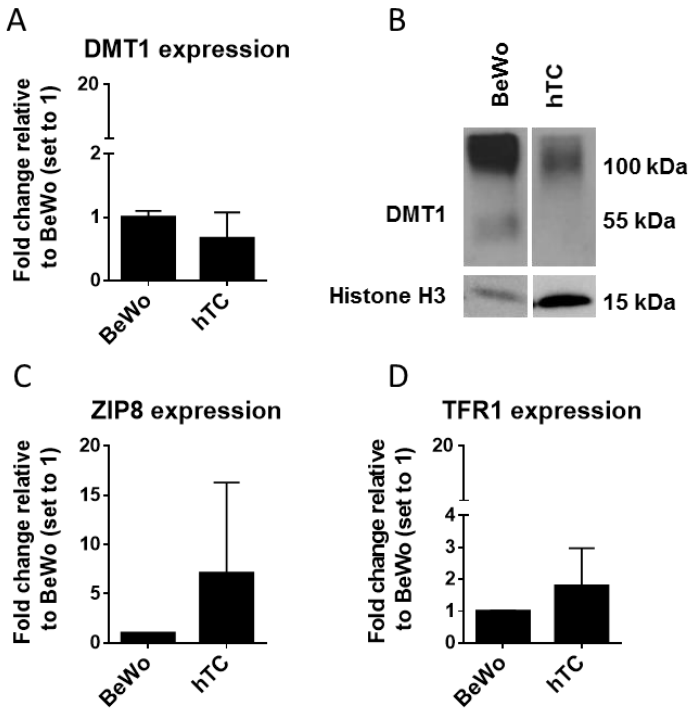

Figure 15. Expression patterns between BeWo and hTCs. Expression patterns between BeWo cells and human trophoblast cells (hTCs) were analysed for DMT1 (A, B), ZIP8 (C) and TFR1 (D). Bar graphs represent mean ± SD from two (BeWo) to four (hTCs) independent experiments.

To establish whether these alterations in gene expression patterns are linked to the differentiation status of the cells, BeWo cells were treated with Forskolin over a period of 72 hours. RNA was isolated from these cells at certain time points (24h, 48h, 72h) and changes in DMT1, ZIP8 and TFR1 expression were analysed by quantitative PCR. DMT1 expression decreased in Forskolin-treated cells in a time dependent manner (Figure 16A), while ZIP8 mRNA levels increased upon Forskolin treatment (Figure 16B). Furthermore, TFR1 levels were also elevated by Forskolin, both on the mRNA level (Figure 16C) and on the protein level (Figure 16D).

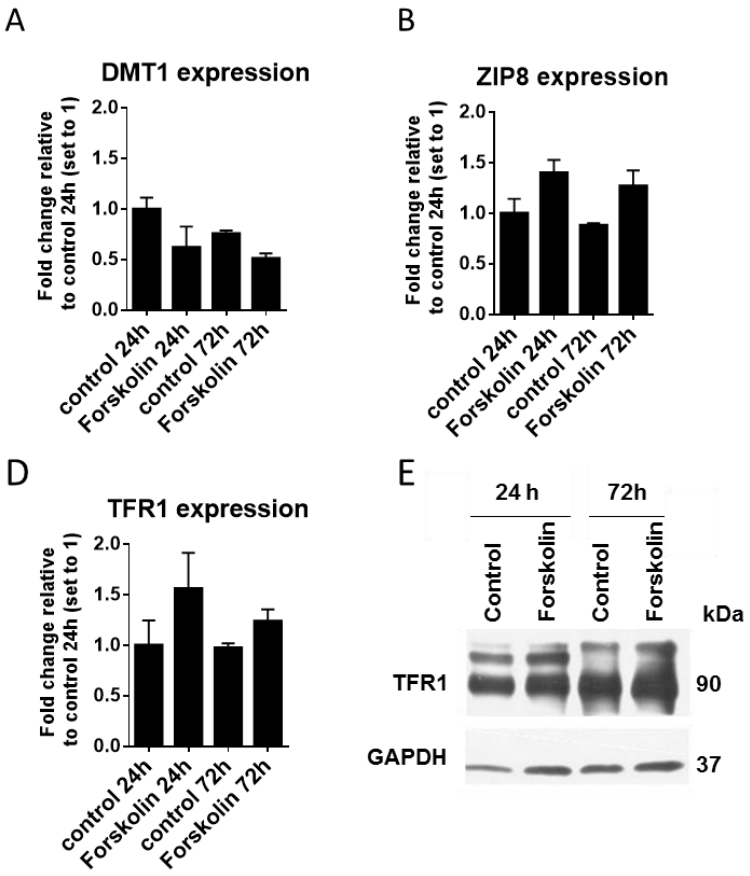

Figure 16. Forskolin treatment of BeWo cells. BeWo cells were treated with Forskolin or DMSO (control) for 24 and 72 hours. Expression of DMT1 (A), ZIP8 (B) and TFR1 (C, D) was analysed. Data represent mean ± SD from three independent experiments.

4. Discussion

Small-interfering RNA has been employed as a potent biotechnological tool for over a decade to gain information about the function of certain gene products [120]. In this study, siRNA mediated gene knockdown was used to elucidate the functional involvement of iron transporters TFR1, DMT1 and ZIP8 in the placental iron metabolism. The aim was to simulate the *in-vivo* situation as closely as possible. Therefore cells were not iron depleted with chelators like deferoxamine (DFO), followed by incubation with either TBI or NTBI [82], because DFO is known to interfere with the proliferation of many cell lines [121].

In knockdown experiments also the specificity of the employed antibodies was validated [122]. Reduced bands for TFR1 at 90 kDa (at higher resolving gels two bands between 70 and 100 kDa (Figure S1) that are probably differently modified [123] monomers) and 170 kDa (dimeric receptor) (Figure 6D) and for DMT1 at 55 kDa as well as 70-100 kDa (Figure 10B) were observed upon the respective knockdown treatment that were in accordance with previous reports on their molecular sizes [124], [125]. Interestingly, the larger monomer band for TFR1 increased its molecular size upon 72 h Forskolin treatment as well as in the corresponding DMSO-treated control group (Figure 16D). It is known that DMSO can induce cellular differentiation via protein kinase C (PKC) pathways [126]. Therefore, it is conceivable that TFR1 is post-translationally modified by a downstream target of PKC upon DMSO treatment.

In contrast, the employed ZIP8 antibody showed many bands ranging from 55-170 kDa (Figure 13E), which can be attributed to different post-translational modifications of the protein [127]. To determine the most prominent immunoreactive band, ZIP8 KD experiments were additionally performed in HeLa cells [127]. In BeWo as well as in HeLa cells a specific region at 130 kDa was reduced in its signal intensity upon ZIP8 knockdown (Figure 13F). This molecular size is in accordance with previous reports [91], [127].

4.1 TFR1 knockdown

TFR1 shows variable tissue expression. Particularly the placenta is characterized by an especially high density of the molecule [128], which has been attributed to the increased requirement of transferrin bound iron, the major iron source for the developing fetus [129]. In contrast, crypt cells decrease TFR1 expression and upregulate DMT1 expression during their differentiation to mature enterocytes. These changes in receptor abundance are a functional adaptation, because in the duodenum nonheme-iron is taken up by DMT1 upon its reduction to the ferrous form [130],

[131]. Nevertheless, extensive studies on TFR1 expression and function in the human placenta have not been performed so far.

Silencing of TFR1 affects cellular iron contents

Since total loss of TFR1 is lethal [84], many different approaches have been employed to down-regulate TFR1 expression in functional studies. Ligand based techniques include intracellular antibodies [132] and small molecule inhibitors [133] that both result in decreased surface expression of the receptor. Alternatively, siRNA mediated gene knockdown has been used to determine the effects of TFR1 diminishment [134], [135]. As expected, all these studies reported decreased cellular iron levels upon silencing of the receptor.

It is therefore of high interest that TFR1 knockdown in BeWo cells unexpectedly resulted in an increase in cellular iron levels (Figure 6B). Furthermore, the strength of this effect depends on the activity of residual TFR1 molecules (Figure 6F). However, it should be noted that due to the great efficiency of the employed siRNA, only relatively high knockdown efficiencies (70-93%) were achieved. Therefore, it would be interesting to use different siRNA concentrations ranging from 50-6.25 nM siRNA (100%-12.5% of the amount in the original protocol [119]) to determine whether the linear dependency of TFR1 abundance and cellular iron levels also occurs at lower knockdown efficiencies.

The presence of increased cellular iron levels upon TFR1 silencing were also indirectly indicated by elevated expression of the iron regulated proteins FPN1 (Figure 7B) and FTH1 (Figure 7C). When cellular iron levels rise, expression of FPN1 and FTH1 increases to efflux or to store the excessive iron. FPN1 and FTH1 expression levels are mainly regulated on the post-transcriptional level via de-repression of mRNA translation under iron-rich conditions by the IRE/IRP system [136]. However, under conditions of high oxidative stress (which can be caused by excessive iron levels) also transcriptional upregulation has been demonstrated for both proteins. The enhanced gene expression of FPN1 and FTH1 is mediated by transcription factor nuclear factor-E2-related factor 2 (Nrf2) to prevent oxidative damage [137], [138].

Unfortunately, total cellular iron levels are seldom measured in *in vitro* studies. In a report on osteoblastic cells, cellular iron was quantified by a spectroscopic approach using confocal laser scanning microscopy. The FPN1 mRNA levels were clearly elevated in iron-rich cells [139]. In contrast, a recent publication claimed that FPN1 mRNA levels in BeWo cells were upregulated upon iron deficiency induction by DFO. For all that is has to be considered that the authors explicitly state that cellular iron levels were not analysed in this study [105]. Furthermore, it has been demonstrated that DFO can induce the upregulation of hypoxia-inducible factor (HIF)-2α

[140]. HIF-2α has been shown to enhance the expression of all FPN1 isoforms [141]. Therefore, the observed increase in FPN1 mRNA upon TFR1 knockdown may be a side effect of DFO treatment.

Far less is known about the iron dependent transcriptional regulation of ferritin. Although previous reports have stated that FTH1 mRNA levels are unaffected by the cellular iron status [142], a recent study showed with a more sensitive detection method that a slight increase in FTH1 transcript copy number occurs upon iron treatment [143]. This result is accordance with the findings of the present study that TFR1 deficient BeWo cells possess higher iron levels as well as higher FTH1 transcripts levels than their controls.

The present findings raise the question on the alternative mechanisms of iron acquisition in BeWo cells. Although transferrin bound iron is the most important iron source for the human placenta, uptake of NTBI, heme iron and ferritin iron has also been demonstrated [79]. Nevertheless, due to high transferrin levels in cell culture medium supplemented with FBS (1.8-2.2 mg/ml transferrin) [144], it can be assumed that TBI is also the main iron source in the present *in vitro* studies on BeWo cells. In future experiments, the TFR1 knockdown experiments should be performed using serum-supplemented medium and medium that contains either exclusively TBI or NTBI to evaluate to what extent iron uptake changes upon knockdown.

The most likely explanation for increased iron accumulation in TFR1 deficient BeWo cells seems to be the moonlighting activity of another molecule. The mRNA expression patterns for the most reasonable candidates DMT1, ZIP8 and ZIP14 did not change upon TFR1 KD (Figure 7D-7F). In parallel to unchanged ZIP8 and ZIP14 levels, also the levels of the associated ferrireductases were not altered (Figure 7G-7H). Nevertheless, it is still conceivable that the cellular localization of any of these transporters might change in response to TFR1 deficiency. For example, it has been shown that DMT1 is preferentially localized at the apical surface of mucosal villi of iron-deficient mice [145]. In addition, ZIP8 can also change its site inside the cell in response to iron conditions. Treatment with high levels of NTBI (FeNTA) resulted in enrichment of ZIP8 at the cell surface. Concomitantly, the levels of TFR1 were decreased as a consequence of high iron levels [91]. It is therefore possible that the cell interprets the reduction of TFR1 as a signal to switch to a mechanism for uptake of NTBI. The most likely candidate for such a mechanism in the human placenta is ZIP8, since placental tissue expression of ZIP8 is much higher than for ZIP14 [91] and its important involvement in NTBI uptake has been demonstrated in a previous [91] as well as in the current study (Figure 13D).

Interestingly, Chinese hamster ovary (CHO) cells can take up TBI independent of TFR1 [146]. Additionally, the surface localization of GAPDH in certain macrophages is controlled by iron availability [147]. By combining these two observations, a recent publication suggested a TFR1 independent pathway for TBI accumulation that is exerted by GAPDH. Under iron depletion global GAPDH synthesis increased and the enzyme strongly localized at the cell surface to serve as a transferrin binding site. Kinetic studies revealed that it is a low affinity, high capacity mechanism [148]. This would be in accordance with the present results insofar as TFR1 deficient cells had even higher iron levels than the controls. This suggests a mechanism that goes beyond a compensatory role. Nevertheless, it cannot be excluded that more than one compensatory mechanism is induced by the loss of TFR1.

It has to be mentioned that in case of TFR1 deficient BeWo cells no increase in total GAPDH synthesis could be observed, also not by densitometry analysis. It is possible that BeWo cells possess enough GAPDH for such a mechanism without the need for further synthesis, since it has been shown that the enzyme is overexpressed in placental cancer cells and cancer cells in general [149].

A very fast and easy method to identify a potential compensatory transporter, would be to analyse changes in the surface localisation of the most likely candidates ZIP8, ZIP14 and GAPDH by cell-surface biotinylation upon TFR1 KD [88]. Any of the target that is enriched in the membrane fraction, could then be easily detected by antibody-based flow cytometry or fluorescence microscopy. Furthermore, double knockdown studies on TFR1 and the putative candidate protein could reveal the functional aspect of the proposed compensatory mechanism.

TFR1 KD experiments in primary trophoblasts

The cancer cell line BeWo [113] has restricted significance for modelling the *in vivo* situation in the human placenta. Especially for studies on iron metabolism immortalized cancer cell lines are rather unsuited, since they show deviant expression of iron transporters (high TFR1 and low FPN1 levels) that ensure that enough iron is available for their increased growth demands [150]. In accordance, the expression patterns for the transporters investigated in this study differed between BeWo cells and primary trophoblasts. While BeWo cells were characterized by higher levels of DMT1, primary trophoblasts exhibited higher amounts of ZIP8 and TFR1 (Figure 15A-D). However, it could be possible that this discrepancy is connected to the state of cellular differentiation. Primary trophoblasts were harvested upon cultivation for approximately 72 hours, which gives them enough time to differentiate into a multinucleated syncytium [151]. If BeWo cells were treated with Forskolin, a drug used to trigger fusion and syncytial differentiation

[152], the expression of DMT1, ZIP8 and TFR1 resembled more the results of primary trophoblasts (Figure 16A-E).

It was necessary to confirm the results on TFR1 knockdown obtained from BeWo cells in human primary trophoblast cells isolated from healthy term placentas. As a matter of fact, cultured trophoblasts cease proliferation and are therefore very hard to transfect [112]. Although an advanced transfection protocol for human primary trophoblast cells [118] was applied, TFR1 knockdown was inefficient in two out of three experiments (Figure 9C,9F). According to the low knockdown efficiency, also the iron content of these cells did not change (Figure 9A,9D). However, in one experiment the knockdown was efficiently reducing TFR1 protein levels (Figure 9I). This again (as in BeWo cells) led to highly increased cellular iron levels (Figure 9G). The data strongly suggest that iron content of trophoblast cells also rise when TFR1 levels decrease.

Silencing of TFR1 affects cell number

Another highly interesting observation in respect to the TFR1 knockdown is the significantly altered number of BeWo cells (Figure 6A). This seemed to be a BeWo specific phenomenon, as cell number in trophoblasts (indicated by their protein content) did not differ between TFR1 knockdown and control treatment. In contrast to the expectation that TFR1 silencing would result in reduced iron uptake, which in turn would reduce cell proliferation, TFR1 deficient BeWo cells accumulated even more iron and were nevertheless substantially reduced in numbers. This finding urges for an explanation.

Due to their already elevated iron demands, cancer cells are very sensitive to additional iron. The present observation that BeWo cell numbers are diminished to about a third upon TFR1 down-regulation suggests that the cells are no longer able to compensate for the high oxidative stress induced by increased iron levels and eventually die by ferroptosis, an iron-dependent form of non-apoptotic cell death [18]. To determine whether ferroptosis is induced by TFR1 knockdown in BeWo cells, two important enzymes of the glutathione system (GCLM, GPX1) were further investigated.

The tripeptide glutathione (GSH) is a major cellular anti-oxidant that is generated in an ATP-dependent two-step reaction. In the first rate limiting reaction, glutamate cysteine ligase (GCL) ligates glutamate and cysteine to form γ-glutamlycysteine (γ-GC). GCL is composed of a catalytic (GCLC) and a regulatory modifier subunit (GCLM). Afterwards, glutathione synthetase (GS) ligates glycine to γ-GC thereby forming GSH. The synthesis of glutathione is regulated by a feedback mechanism that inhibits GCL activity under glutathione-rich conditions. By means of glutathione peroxidase (GPX) the tripeptide is oxidized from GSH to GSSG to detoxify reactive

oxygen species. Oxidized glutathione can be recycled back to GSH by glutathione reductase [153], [154].

In comparison to the control group, TFR1 deficient BeWo cells showed decreased GCLM (Figure 7I) and increased GPX1 (Figure 7J) mRNA levels. Studies in *Gclm* (-/-) knock out mice revealed an essential role for the protein in glutathione synthesis, as GSH levels declined to 9% of the wild-type mice. It was further shown that GCLM promotes glutathione production by decreasing the K_m (Michaelis constant) for ATP and glutamate and by increasing the K_i (dissociation constant) for GSH mediated feedback inhibition as well as the K_{cat} (turn-over number) for γ-GC synthesis [155]. A recent publication claimed that in the case of cysteine deprivation GCLM mRNA and protein are upregulated in order to sustain appropriate levels of GSH synthesis [154]. Since the observed GCLM downregulation in TFR1 deficient BeWo cells might have been caused by enhanced GSH levels, the total amount of glutathione was measured. Although not statistically significant, a clear trend for elevated levels of glutathione was observed in the TFR1 knockdown cells in comparison to controls (Figure 8A).

Taken together the results obtained from the present study could suggest a mechanism, where TFR1 deficiency in BeWo cells induces an alternative, high capacity pathway for iron uptake that concomitantly exposes the cell to increased oxidative stress. Consequently, the cells could go through a selection process in which survival requires:

1) decrease of free iron levels (either by storage in ferritin or export by ferroportin)

2) increase of anti-oxidative protection (high levels of glutathione and glutathione peroxidase to detoxify ROS).

Importantly, no clear statement about involvement of ferroptosis can yet be made, which demands the analysis of ferroptotic marker proteins [22] in future experiments in both BeWo and primary trophoblast cells. Studies in rats have shown that during pregnancy iron is preferentially transported to the fetus, even if this causes anemia in the mother by draining her iron stores [156]. Due to this high priority to ensure optimal fetal iron supply, it might be possible that trophoblastic cells are able to adapt their iron acquisition properties by all means to fulfil these requirements. Since BeWo cells have a trophoblast origin, they might be able to execute such an alternative mechanism, but are eventually overwhelmed by the increasing iron amounts due to their already altered iron demands.

4.2 DMT1 knockdown

DMT1 is a promiscuous metal transporter that mediates the uptake of several divalent cations including manganese (Mn^{2+}), cobalt (Co^{2+}), copper (Cu^{2+}), nickel (Ni^{2+}) and iron (Fe^{2+}) [157].

Nevertheless, the protein seems to be particularly important for iron metabolism, since DMT1 exhibits a very high substrate selectivity for iron [158] and is regulated by cellular iron levels [159]. Consequently, DMT1 is strongly expressed in iron transporting cells, including intestinal [159] and placental [160] cells. Among the cell lines listed in the *Human Protein Atlas* database, the human placental cancer cell line BeWo is characterized by the highest amount of mRNA coding for DMT1 (http://www.proteinatlas.org/ENSG00000110911-SLC11A2/cell; 16.8.2017 21:44).

It is therefore very interesting that silencing of DMT1 has neither an effect on cell number (Figure 10A) nor an obvious effect on the cellular iron levels (Figure 10B). Similar results were observed in primary trophoblasts (Figure 12A,12D), although a higher number of different placenta samples is required to make a definite statement. Nevertheless, the obtained results are not surprising, since it has been previously reported that DMT1 plays a non-essential role in placental iron transport [86].

Due to the tremendously high levels of DMT1 in BeWo cells it is further possible that the number of remaining molecules is sufficient for proper iron transport. While siRNA-mediated gene silencing was unsuccessful in primary trophoblasts (Figure 12C, 12F), knockdown efficiency was high in BeWo cells (on average 86% on mRNA level). However, the dominant smear of the protein at approximately 70-100 kDa (Figure 10D), prevented a quantitative evaluation via densitometry. This smear is most probably caused by different post-translational modifications that influence the molecular weight of the protein [161].

Interestingly, also upon DMT1 silencing the FTH1 levels were significantly increased (Figure 11B). Contrary to the expectation of a compensatory upregulation, mRNA levels of ZIP8 and ZIP14 (Figure 11D-11E) were downregulated; this effect was statistically significant only in case of ZIP14. For both proteins is has been demonstrated that their mRNA levels are not affected by cellular iron levels. However, their protein abundance increases with iron loading presumably via post-transcriptional regulation [91], [162].

It is conceivable that either ZIP8 and/or ZIP14 could compensate for DMT1 loss. Although both proteins can transport iron as well as zinc, ZIP8 has a higher substrate specificity for zinc, while ZIP14 preferentially transports iron. Furthermore, their iron transport activity is pH-dependent and ZIP14 can transport more iron under acidic conditions (down to pH 6.5) than ZIP8 [91], [163]. Therefore, ZIP14 would be best suited to transport iron across the acidified endosome similar to DMT1. If ZIP14 is upregulated to compensate for DMT1 loss, cellular zinc levels might also rise as a side effect. This is of special interest, since the mRNA of some ZIP family

members has been described to be upregulated under low zinc conditions and downregulated during zinc overload [164], [165].

Therefore, the observed decrease in ZIP8 and ZIP14 mRNA in BeWo cells could be the aftermath of increased cellular zinc concentrations, because of the higher import capacity of the respective transporters in response to DMT1 deficiency. The easiest way to test this hypothesis would be to additionally determine cellular zinc levels. Unfortunately, all attempts to do so have failed so far. The zinc background concentrations in the solvents were too high for measurements via GF-AAS, but samples values were far too low for analysis by flame-AAS. An alternative - albeit indirect- approach to evaluate cellular zinc levels, would be the characterization of metallothionein expression, since it should increase at higher amounts of the metal [166].

4.3 ZIP8 knockdown

There is particular evidence that ZIP8 is involved in iron transport across placental cells. ZIP8 deficient BeWo cells showed a 40% reduction in the uptake of radioactively labelled iron in comparison to control cells [91].

In the presence of serum-supplemented medium, ZIP8 silencing had neither an effect on cell numbers nor on cellular iron levels (Figure 13A-B). Companies usually do not provide information on the transferrin concentration of serum supplements. Nonetheless, since transferrin is an abundant serum protein, it can be assumed that most of the iron is present as TBI [167]. From that perspective (TFR1 is active and sufficient amount of TBI is available), it is no surprise that ZIP8 knockdown did not affect cellular iron accumulation.

In a next step, ZIP8 deprived cells were incubated to ferric ammonium citrate (NTBI) as sole iron source [91]. This treatment led to a quite similar result, namely 44% reduction in iron uptake (Figure 13D) compared to 40% reported by Wang et al. [91]. These findings strongly suggest that ZIP8 mediates uptake of NTBI into placental cells.

In general, a big role of ZIP8 in placental iron uptake is questionable. ZIP 8 is unsuited for the transport of TBI [91] but maternal blood provides only TBI (and no or negligible amounts of NTBI) to placental cells.

Interestingly, the same study that showed reduced tissue iron levels (liver, lung and kidney) in the pups of hypomorphic *Slc39a8* mice also found a simultaneous decrease in zinc levels. This zinc deficiency might have contributed to the observed impaired development of the offspring just as well. Although ZIP8 seems to be indispensable for embryo development in general, placenta-specific knockout mice will be required [90]. A recent publication on prenatal micronutrient supplementation in pregnant women describes a decrease in placental ZIP8 mRNA expres-

sion upon appropriate zinc supply [168] suggesting that the primary role of ZIP8 in the human placenta might be the uptake of zinc.

Also the results from ZIP8 deprived BeWo cells have to be confirmed in primary trophoblasts. It would be very interesting in this context to relate the ZIP8 expression levels of freshly isolated trophoblasts to placental and maternal zinc status.

5. Conclusion

5.1 Summary

Despite good knowledge of mammalian iron metabolism in general, surprisingly little is known about the specifics of placental iron metabolism and transfer. Although studies on human placental iron metabolism are required to broaden our knowledge on this subject, they are unfortunately very rare. Therefore, this Master thesis aimed to elucidate molecular mechanisms that are involved in iron uptake into the placenta. The functional role of the iron transporters TFR1, DMT1 and ZIP8 was examined using siRNA mediated gene knockdown. Experiments were mainly performed in the human choriocarcinoma cell line BeWo, since it is a well-established *in vitro* model for studies on the human placental iron metabolism. One remarkable feature of the present study is the fact that in addition to analysing regulatory interactions, cellular iron levels were also quantitatively determined by GF-AAS. It could be demonstrated that DMT1 and ZIP8 are dispensable for iron accumulation in BeWo cells under TBI-rich conditions. However, ZIP8 might be very important for the uptake of NTBI into placental cells. The most striking finding was the increase in cellular iron levels upon silencing of TFR1. Furthermore, TFR1 knockdown in the BeWo model resulted in decreased cell number and upregulation of genes required to ameliorate the damage of oxidative stress by either reducing free iron (FPN1, FTH1) or detoxifying ROS (GPX1). Several hypotheses for a compensatory mechanism underlying this phenomenon have been proposed above (for a summary see Figure 17).

Additionally, the functional studies were also performed in primary trophoblast cells. Quite similar results as in BeWo cells could be observed. Nevertheless, more studies in primary trophoblast cells are necessary due to their high biological variability [169].

In conclusion, these initial experiments provided important first insights into the functional role of the tested iron transporters, which will help in future transport studies.

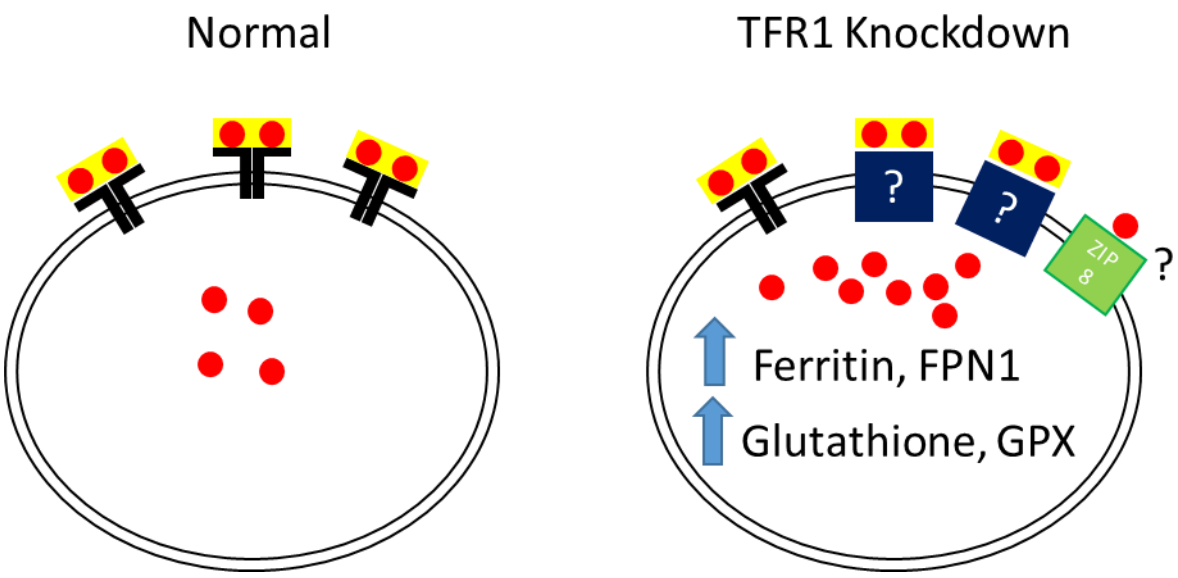

Figure 17. Model for TFR1 deprived BeWo cells. In normal BeWo cells (left ellipse) transferrin bound iron (yellow rectangles represent transferrin; red dots represent iron independent of its oxidative state) is taken up by Transferrin Receptor 1 (black T-shape). Upon TFR1 knockdown (right ellipse), the cell may recruit a so far unknown transferrin importer to the cell surface to allow ongoing transferrin-iron up-take. Alternatively, also the localization of ZIP8 could be altered to allow up-take of NTBI. Upregulation of Ferritin, FPN1, glutathione and GPX may protect the cell from additional oxidative damage caused by these high iron amounts.

5.2 Outlook

5.2.1 Transport studies

All experiments of the present study were conducted in conventional 2D cell culture plates, which allows the iron atoms to go through several cycles of import and export. This might have biased the study results. In order to study 'real' iron transport, it will be necessary to adapt the experimental protocol to the conditions in the transwell assay. The BeWo system is well suited for such application [170]. Studies on iron uptake and iron efflux in BeWo cells deficient for a certain transporter can be expected to provide important insight into iron traffic across the human placenta.

Furthermore, the current protocol used for quantification of low iron concentrations must be improved. The wet acid digestion procedure is highly susceptible to contaminations [171]. Although ultrapure nitric acid has been used in the present study, the background iron level was quite high. Sample specific signals were therefore close to the background noise. The mean recoveries of the reference material were relatively low (77% and 80%) probably originating from the high dilution factor (1:10,000) that has to be applied to reach the linear absorption range given for GF-AAS iron analyses. It has to be mentioned that analyses of the reference material with flame-AAS (dilution is 1:5 or 1:10; no background) yields a perfect recovery of ≥98%.

As iron naturally occurs in cells, radioactively labelled iron has to be employed to distinguish exogenous from endogenous iron [111]. An appealing alternative would be the use of non-radioactive iron isotopes, whose transport can be followed by ICP-mass spectroscopy [172].

5.2.2 Focus on primary cells

Cell lines have been extensively used in basic research for many decades. Although cost-efficient and easily available, they bear the risk of cross-contamination and misidentification. An even greater problem is the accumulation of mutations and promotion of the genetic drift with increasing passaging number [173]. Since primary cells come closest to the *in vivo* situation they should be the model of choice. Nevertheless, several experimental parameters have to be optimized in this regard. At the moment, the biggest problem is the low knockdown efficiency in primary trophoblasts, since siRNA-mediated gene silencing was rather unsuccessful so far. A promising alternative would be the use of small hairpin (sh)RNA constructs delivered by viral vectors, which is most effective for freshly isolated trophoblasts [174]. In general establishing this technique would be highly interesting, since it could generate stable knockdown BeWo lines that could be used in future transport studies.

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Appendix

Supplementary Table 1: **Materials for siRNA-mediated gene knockdown**

Product	Company, Article nr.
Lipofectamine RNAiMAX	Life Technologies, #13778
OptiMem	Gibco, #319850
ON-TARGETplus Non-targeting Pool	Dharamacon, D-001810-10-20
SMARTpool: ON-TARGETplus human SLC11A2	Dharmacon, L-007381-00-0005
SMARTpool: ON-TARGETplus human SLC39A8	Dharmacon L-007573-00-0005
SMARTpool: ON-TARGETplus human TFRC	Dharmacon, L-003941-00-0005
5x siRNA buffer	Dharmacon, B-002000-UB-100

Supplementary Table 2**: List of antibodies**

Product	Company, Article nr.
Primary antibodies (diluted 1:1000)	
Anti-α-tubulin mouse monoclonal, clone DMA1	Merck Millipore, #05-829
Anti-Cytokeratin-7 rabbit monoclonal	Cell Signaling Technology, #4465
Anti-DMT1 (SLC11A2)	Cell Signaling Technology, #15083
Anti-GAPDH rabbit polyclonal	Trevigen, #2275-PC-100
Anti-Histone H3 rabbit monoclonal	Cell Signaling Technology, #449
Anti-TFR1/CD71 (TFRC)	Cell Signaling Technology, #13113
Anti-ZIP8 (SLC39A8)	Proteintech, USA, #20459-1-AP
Secondary antibodies (diluted 1:10.000)	
Mouse IgG-heavy and light chain antibody	Bethyl Laboratories,A90-116P
Rabbit IgG-heavy and light chain antibody	Bethyl Laboratories,A120-101P

Supplementary Table 3: **List of primers**

Target gene	Company, Assay ID
CYBASC3	Thermofisher Scientific, Hs00328512_m1
FTH1	Thermofisher Scientific, Hs01000477_g1
GCLC	Thermofisher Scientific, Hs00155249_m1
GCLM	Thermofisher Scientific, Hs00157694_m1
GPX1	Thermofisher Scientific, Hs00829989_gH
GSR	Thermofisher Scientific, Hs00167317_m1
SLC11A2	Thermofisher Scientific, Hs00167206_m1
SLC39A8	Thermofisher Scientific, Hs01061802_m1
SLC39A14	Thermofisher Scientific, Hs00299262_m1
SLC40A1	Thermofisher Scientific, Hs00205888_m1
STEAP3	Thermofisher Scientific, Hs00217292_m1
STEAP4	Thermofisher Scientific, Hs01026584_m1
TBP	Thermofisher Scientific, Hs00427620_m1
TFRC	Thermofisher Scientific, Hs00174609_m1
UBC	Thermofisher Scientific, Hs00824723_m1

Supplementary Table 4. **List of chemicals and buffers**

Acrylamide	30% Acrylamide/bis-Acrylamide (Sigma)
APS	10% Ammoniumpersulfat (Sigma)
Bradford (5X)	Bradford Protein Assay (Bio-rad)
Buffer B	1.5 M Tris (Sigma); 0.4% SDS (VWR); pH 8.8
Buffer C	0.5 M Tris (Sigma); 0.4% SDS (VWR); pH 6.8
DMSO	Dimethylsulfoxid (Sigma)
Electrophoresis Buffer (10X)	1.5 M Tris (Sigma); 1.95 M Glycine (VWR); 20% SDS (VWR)
Forskolin	Forskolin from *Coleus forskohlii* (Sigma)
Harlow Buffer	1.5 M Tris (Sigma); 0.4 M Glycine (Sigma); 20% SDS (VWR); Methanol (Sigma)
Loading dye (4X)	200 mM Tris pH 6.8 (Sigma); 400 mM DTT (Bio-Rad); 8% SDS (VWR); 0.4% Bromphenolblau (Merck); 40% Glycerol (Merck)
Lysis Buffer	RIPA buffer (1% PMSF, 2% PIM)
PIM	200µg/ml Leupeptin (Sigma); 200 µg/ml Aprotinin (Sigma); 30 µg/ml Benzamidinchlorid (Sigma); 1000 µg/ml Trypsin inhibitor (Sigma)
PMSF	10 mM Phenylmethylsulfonylfluorid (Sigma) in Isopropanol (Sigma)
Ponceau (10X)	2% Ponceau S, 30% Trichlor acetic acid (Merck), 30% Sulfosalicylic acid (Merck)
Protein Ladder	PageRuler™ Prestained Protein Ladder (Thermo Scientific)
RIPA buffer	50 mM Tris pH 7.6 ;150 mM NaCl (VWR); 1% Triton X-100 (Sigma) ; 0.1% SDS (VWR); 0.5% Sodium deoxycolate (Sigma)
TBS (10X)	1.5 M NaCl (VWR); 0.5 M Tris (Sigma); pH 7.4
TBST	1X TBS; 0.1% Tween 20 (VWR)
TEMED	*N*,*N*,*N*′,*N*′-Tetramethylethan-1,2-diamin (Sigma)

Supplementary Table 5. **SDS-PAGE gel composition**

Reagent	**Running gel (final conc.)**	**Stacking gel (final conc.)**
Acrylamide (30%)	As required	5%
Buffer B	25%	-
Buffer C	-	25%
APS	1%	1%
TEMED	0.83%	0.83%

Protocol: **Cell thawing**

1) Take cells from liquid nitrogen and thaw at room temperature
2) Add 1 ml pre-warmed medium and pipet suspension to 5 ml pre-warmed medium in 15 ml Falcon
3) Centrifuge 3 min at 200 x g
4) Resuspend pellet in 10 ml pre-warmed medium and culture on 10 cm dishes

Protocol: **Cell passaging**

1) Remove medium from 10 cm dish and wash cells with 5 ml DPBS
2) Detach cells with 1 ml 0.25% trypsin/EDTA for 5-7 min at 37°C
3) Inhibit trypsin by addition of 5 ml medium and transfer cell suspension into Falcon
4) Centrifuge 3 min at 200 x g
5) Split cell pellet (usually 1:2 or 1:3)

Protocol: **Cell freezing**

1) Remove medium from 10 cm dish and wash cells with 5 ml DPBS
2) Detach cells with 1 ml 0.25% trypsin/EDTA for 5-7 min at 37°C
3) Inhibit trypsin by addition of 5 ml medium and transfer cell suspension into 15 or 50 ml Falcon
4) Centrifuge 3 min at 200 x g
5) Resuspend cell pellet in 900 µl FCS
6) Transfer suspension into cryotubes
7) Add dropwise 100 µl DMSO
8) Incubate 20 min on ice
9) Keep at -80°C over night
10) Long-term storage in liquid nitrogen

Protocol: **Cell harvest (general)**

1) Incubate cells on ice for 5 min (all steps are performed on ice from this point onwards)
2) Remove medium and collect in 15 ml Falcons
3) Spin down medium at 1800 x g and transfer 1 ml supernatant into fresh tube
4) Wash cells with cold DBPS (same amount as medium)
5) Detach cells with 0.25% trypsin/EDTA (1/6 of the volume of the culture medium) for 5-7 min at 4°C

6) Stop reaction by adding 5x volume of culture medium

7) Transfer cell suspension into fresh tube

8) Centrifuge 3 min at 200 x g

9) Resuspend pellet in 1 ml cold DPBS

10) Add 50 µl cell suspension to 10 ml CASYton Buffer (1:200) and determine cell number

11) Pool triplicates and centrifuge 3 min at 200 x g

12) Resuspend pellet in 1 ml cold DPBS

Optional: Resuspend pellet 1 in 1 ml cold DPBS (50 µM DTPA) and incubate on ice for 3 min

13) Centrifuge 3 min 200 x g

14) Resuspend pellet in 1 ml cold DPBS and aliquot as required

15) Centrifuge 3 min 200 x g

16) Remove supernatant and store pellets -20°C

Protocol: **Cell harvest (Human primary trophopblasts)**

1) Incubate cells on ice for 5 min (all steps are performed on ice from this point onwards)

2) Remove medium and collect in 15 ml Falcons

3) Spin down medium at 1800 x g and transfer 1 ml supernatant into fresh tube

4) Wash cells with cold DBPS (same amount as medium)

5) Incubate with 1 ml DPBS (50 µM DTPA) for 3 min

6) Detach cells using cell scraper and transfer suspension into fresh tubes

7) Centrifuge for 3 min at 200 x g

8) Resuspend pellet in 1 ml cold DPBS and aliquot as required

9) Centrifuge for 3 min at 200 x g

10) Remove supernatant and store pellets at -20°C

Protocol: **RNA Extraction**

1) Lyse cells in 350 µl Trizol (Sigma) for 5 min at room temperature or over-night at -20°C

2) Add 120 µL nuclease-free water and 100 µL Chloroform and mix by vortexing for 15 s

3) Incubate 10 min at room temperature and vortex again

4) Centrifuge for 15 min at 4°C at 16060 x g

5) Transfer aqueous phase into fresh tube (from this point on all steps are performed on ice)

6) Add 1 µl Glycoblue (Ambion) and 280 µl cold isopropanol and vortex samples

7) Incubate on ice for 30 min

8) Centrifuge for 30 min at 4°C at 16060 x g

9) Remove supernatant and wash with 1 ml 75% cold ethanol

10) Centrifuge for 7 min at 4°C at 16060 x g

11) Remove supernatant and air dry pellet for up to 10 min

12) Dissolve RNA pellet in 10-20 µl nuclease-free water and store at -80°C

Protocol: **Reverse Transcription**

1) Determine RNA concentration (Nanodrop-1000, Thermofisher Scientific)

2) Adjust the amount of RNA to be reverse transcribed

Component	Volume (µl)
RNA	X (usually 1000 ng)
Random primer	1
Nuclease-free water	Fill up to 5

3) Place on preheated 70°C thermocycler (Peqlab) for 5 min, then put on ice for 5 min

4) Add 15 µl reverse transcription mix

Component	Volume (µl)
M-MLV RT 5X Buffer	4
$MgCl_2$ (25 mM)	1.2
PCR Nucleotide Mix (dNTP)	1
Recombinant RNasin Ribonuclease Inhibitor	0.5
M-MLV Reverse Transcriptase (200 u/µl)	1
Nuclease-free water	7.3

5) Incubate in thermocycler: 5 minutes at 25°C, 60 minutes at 42°C, 15 minutes at 70°C

Protocol: **Quantitative Real-time PCR**

1) Prepare working solution by diluting cDNA 1:11 (10 µl cDNA + 100 µl nuclease-free water)

2) Add 2 µl working solution in wells of 96-well plate (in duplets) by reverse-pipetting

3) Add 13 µl PCR master mix

Component	Volume (µl)
Primer	0.75
iTaq Universal Probes Supermix (Bio-rad)	7.5
Nuclease-free water	4.75

4) Spin down for 1 min at 207 x g
5) Read plate using Applied Biosystems StepOnePlus™ Real-Time PCR System: Initiated with a holding stage (50°C for two minutes, denaturation at 95°C for 10 minutes), followed by a cycling stage (for 15 seconds at 95°C and for one minute at 60°C (40x)).

Protocol: **Protein extraction**

1) Lyse cell pellet in 3 times volume of lysis buffer (RIPA (1% PMSF, 2% PIM))
2) Vortex and incubate on ice for 20 min
3) Vortex and centrifuge at 21382 x g for 20 min at 4°C
4) Transfer supernatant into fresh tubes
5) Add 1 µl protein lysat to 1 ml 1X Bradford reagent, incubate for 5 min at RT and determine protein concentration

Protocol: **Western Blot**

1) Boil samples for 10 min at 72°C
2) Run gel at 70 V (A=max, W=max)
3) Switch to 120V when the dye front reaches the resolving gel
4) Blot 1h at 350 mA (V=max, W=max)
5) Briefly rinse with dH_2O and stain 10 min Ponceau
6) Wash 3 x 10 min TBST
7) Block for 1 h in TBST (5% milk)
8) Wash 3 x 10 min TBST
9) Incubate with primary antibody (diluted as described above in TBST (5% BSA)) over night at 4°C
10) Wash 3 x 10 min TBST
11) Incubate with secondary antibody (diluted as described above in TBST (5% milk)) for 1h at RT
12) Wash 3 x 10 min TBST
13) Incubate blots with ECL solution for 1 min and develop films

Protocol: **GF-AAS measurements**

1) Transfer 1 ml medium or suspension into 10 ml MARSXpress Vessel (PFA)
2) Add 1.5 ml HNO_3 (Roth, Ultrapure)

3) Digest in MARS 6 microwave oven (CEM) at 200°C (20 min heating to 200°C, 15 min at 200°C, cool down to 80°C)
4) Incubate vessels at 4°C for 15 min
5) Remove lid and let evaporate for 15 min
6) Transfer content into acid-washed PE-tube
7) Rinse vessel with 1 ml H_2O and transfer into same PE-tube
8) Note weight and measure (or store at 4°C)

Higher resolution immunoblot of TFR1

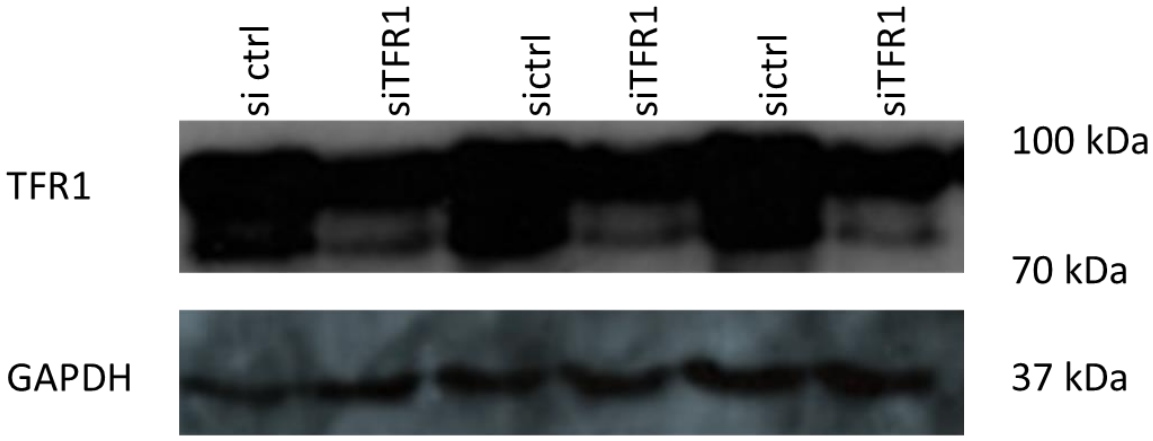

Figure S1. TFR1 bands. Using a higher resolving gel (7.5%) the specific band for TFR1 at 90 kDa could be further separated into two bands between 70-100 kDa.

Abstract

Iron is an essential trace element that is required by all cells, but toxic when present in excess. Iron deficiency is the most common form of micronutritional deficiency. Infants as well as pregnant women are most likely to suffer from it. During pregnancy, the placenta is the major interface of nutritional exchange between mother and developing fetus. Although mammalian iron metabolism is in general very well characterized, surprisingly little is known about iron metabolism and transfer in the human placenta.

In this Master thesis the functional role of iron transporters Transferrin receptor (TFR)1 (*TFRC*), Divalent Metal Transporter (DMT)1 (*SLC11A2*) and ZRT/IRT like protein (ZIP)8 (*SLC39A8*) in iron uptake was investigated by siRNA-mediated gene knockdown in human choriocarcinoma cell line BeWo and in primary trophoblasts isolated from three healthy term placentas. Upon knockdown, changes in cellular iron levels relative to controls (treated with non-targeting siRNA) were analysed using graphite furnace atomic absorption spectroscopy (GF-AAS). Additionally, gene expression of eleven proteins involved in iron and glutathione metabolism (TFR1, Ferroportin (FPN)1, Ferritin heavy chain (FTH)1, DMT1, ZIP8, ZIP14, Six trans-membrane epithelial antigen of the prostate (STEAP)3, Cytochrome b ascorbate-dependent protein (CYB561A)3, Glutamate cysteine ligase modifier subunit (GCLM), Glutathione peroxidase (GPX)1, Glutathione reductase (GR)) were determined by quantitative PCR.

The knockdown experiments indicated that DMT1 and ZIP8 seem to be dispensable for iron uptake in BeWo cells under conditions of high transferrin-bound iron availability. However, ZIP8 is important for the transport of non-transferrin bound iron (NTBI) as BeWo cells accumulate significantly less iron ($P<0.05$) when only NTBI (ferric ammonium citrate) is offered to cells. Interestingly, TFR1 knockdown resulted in significantly increased cellular iron levels accompanied by decreased cell numbers ($P<0.05$). In line with this, FPN1 and FTH1 as well as GPX1 known to protect cells from excessive iron and oxidative stress were upregulated. Similar results on cellular iron acquisition as in BeWo cells could be observed for primary trophoblasts. Nonetheless, more experiments on primary trophoblasts are required to confirm the findings.

In conclusion, the experiments provide first insights into the function of TFR1, DMT1 and ZIP8 in the uptake of iron into human placental cells. Further studies are needed to elucidate the complex regulation and interaction of the proteins.

Zusammenfassung

Eisen ist ein wichtiges Spurenelement, das von allen Zellen benötigt wird, jedoch toxisch im Überschuss ist. Eisendefizienz ist die weltweit häufigste Form einer Mikronährstoffdefizienz. Am häufigsten sind Kleinkinder und schwangere Frauen von dieser betroffen. Während der Schwangerschaft fungiert die Plazenta als Schnittstelle des Nährstoffaustausches zwischen Mutter und Kind. Obwohl der Eisenmetabolismus in Säugetieren generell sehr gut charakterisiert ist, ist erstaunlich wenig über den plazentalen Eisenmetabolismus bekannt.

Im Laufe dieser Masterarbeit wurde die funktionelle Rolle der Eisentransporter Transferrin Receptor 1 (TFR)1 (*TFRC*), Divalent Metal Transporter (DMT)1 (*SLC11A2*) und ZRT/IRT like protein (ZIP)8 (*SLC39A89*) für die Eisenaufnahme der humanen Chorionkarzinomazelllinie BeWo, sowie primärer Trophoblasten, welche aus gesunden Plazentas isoliert worden waren, mittels siRNA vermittelter Herunterregulierung der Genexpression untersucht. Änderungen der zellulären Eisenlevel als Konsequenz verminderter Transporterexpression wurde mittels Graphit-Ofen-Atomabsorptionsspektrometrie analysiert. Zusätzlich wurden auch Änderungen in der Genexpression von elf Proteinen, welche in den Eisen- sowie den Glutathionmetabolism involviert sind (TFR1, Ferroportin (FPN)1, Ferritin heavy chain (FTH)1, DMT1, ZIP8, ZIP14, Six transmembrane epithelial antigen of the prostate (STEAP)3, Cytochrome b ascorbate-dependent protein (CYB561A)3, Glutamate cysteine ligase modifier subunit (GCLM), Glutathione peroxidase (GPX)1, Glutathione reductase (GR)) mittels quantitativer Polymerasekettenreaktion verfolgt.

Unter ausreichender Verfügbarkeit von transferrin-gebundenem Eisen schien die Herunterregulierung von DMT1 und ZIP8 keine Auswirkung auf die Eisenaufnahme in BeWo Zellen zu haben. Wurden die BeWo Zellen jedoch ausschließlich mit nicht-transferrin gebundenem Eisen (in der Form von ferrischen Ammoniumcitrat) kultiviert, haben sie deutlich weniger Eisen nach Herunterregulierung von ZIP8 aufgenommen ($P<0.05$). Interessanterweise resultierte verminderte Expression von TFR1 in erhöhten zellulären Eisengehalten und reduzierter Zellzahl ($P<0.05$). Um den Gefahren des überschüssigen Eisens zu entgehen, regulierten die TFR1 defizienten Zellen Gene hinauf, deren Produkte sowohl die Verfügbarkeit freien Eisens reduzieren (FPN1, FTH1), sowie die Bildung freier Radikale verhindern (GPX1). In Bezug auf die zellulären Eisengehalte konnten in den primären Trophoblasten ähnliche Effekte wie in den BeWo Zellen nach Herunterregulierung des jeweiligen Transporters beobachtet werden. Trotzdem müssen noch weitere Experimente in primären Trophoblasten durchgeführt werden.

Zusammenfassend, haben die durchgeführten Experimente erste wichtige Einblicke über die Funktion von TFR1, DMT1 und ZIP8 für den Eisentransport in humanen Plazentazellen gelie-

fert. Weitere Studien sind jedoch gefragt, um ein besseres Bild dieser komplexen Netzwerke zu erlangen.

Curriculum vitae

Education:

Since 2015: Master studies in Molecular Biology (Focus on Biochemistry), University of Vienna, Austria

2011-2015: Bachelor studies in Biology (Focus on Microbiology and Genetics), University of Vienna, Austria

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2003-2011: Sperlgymnasium, 1020 Vienna, Kl. Sperlgasse 2c

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Performance scholarship awarded by the University of Vienna for exceptional curricular achievements (10/2015 – 09/2016)

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C. Balthasar, H. Stangl, **R. Widhalm**, S. Granitzer, M. Hengstschläger, and C. Gundacker, "Methylmercury Uptake into BeWo Cells Depends on LAT2-4F2hc , a System L Amino Acid Transporter," *Int. J. Mol. Sci.*, vol. 18, no. 8, 2017.