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Role of EGFR signaling during epithelial development and tumor formation

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For my Dad,
who left this world far too early

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1 ZUSAMMENFASSUNG

Epidermaler Wachstumsfaktorrezeptor (EGFR)-defiziente Mäuse sterben in unterschiedlichen Stadien der embryonalen und frühen postnatalen Entwicklung und weisen verschiedene epitheliale Defekte sowie eine Neurodegeneration auf. Neben seiner Funktion in der Regulation der normalen Entwicklung von Astrozyten und Epithelzellen wird der EGFR in vielen humanen Karzinomen und Glioblastomen, welche Tumore epithelialen beziehungsweise glialen Ursprungs sind, überexprimiert. Transgene Mäuse, die eine aktivierte Form des Ras-Aktivators Son of Sevenless unter dem Epithel-spezifischen Keratin 5 Promotor exprimieren (K5-SOS), entwickeln EGFR-abhängige Hauttumore. In diesen Tumoren stellt der EGFR ein essentielles Überlebenssignal für die Tumorzellen dar, was vermutlich durch die Aktivierung von Akt erfolgt. Deswegen ist es sehr wichtig, die physiologischen Signaltransduktionskaskaden des EGFR in Epithelzellen genau zu untersuchen, um besser zu verstehen, wie abweichende EGFR Signalisierung zur Tumorbildung und zum Tumorwachstum führen.

Aufgrund der frühen postnatalen Letalität der EGFR-null Mäuse konnte die Funktion des EGFR während der Haut- und Haarfollikelentwicklung nicht untersucht werden. Deshalb wurden Mäuse mit konditionellen EGFR (floxed) Allelen mit der K5-Cre oder der Tamoxifen-induzierbaren K5-CreER^{T} transgenen Linie gekreuzt, um Mäuse zu generieren, in denen der EGFR spezifisch in den basalen Schichten der Epidermis während der Embryogenese (EGFR$^{\Delta ep}$) bzw. in adulten Mäusen (EGFR$^{\Delta epER}$) deletiert werden kann. Überraschenderweise führte die Epidermis-spezifische Inaktivierung des EGFR während der Embryonalentwicklung oder der ersten postnatalen Tage zum frühen Tod. Die Deletion des EGFR in der Haut zu einem späteren Zeitpunkt verursachte hingegen nur geringe Anomalien der Haarfollikel, und die Mäuse waren überlebensfähig. Die Bildung der lebenswichtigen Hautbarriere war in EGFR-defizienten sowie EGFR$^{\Delta ep}$ Mäusen verzögert, wobei die Haut kurz nach der Geburt impermeabel wurde.

Des Weiteren konnte ich zeigen, dass das Fehlen des EGFR in der Epidermis die Morphogenese der Haarfollikel sowie deren Eintritt in den Haarzyklus verzögert. Darüber hinaus spielt der EGFR offenbar eine Rolle in der

Immunantwort der Haut, da Langerhans Zellen, αβT-Zellen, dendritische Zellen, Granulozyten und Mastzellen die Haut von EGFR-mutanten Mäusen infiltrieren unabhängig davon, ob Haarfollikel vorhanden sind oder nicht. Meine Studien haben auch gezeigt, dass in der Abwesenheit des EGFR die Wundheilung verzögert ist, da die Reepithelialisierung und Wundkontraktion beeinträchtigt sind.

Neben der essentiellen Funktion in der Entwicklung und Erhaltung der Haut und ihrer Anhänge ist der EGFR in die Hauttumorigenese involviert. Wie bereits erwähnt, stellt der EGFR im K5-SOS-abhängigen Tumormodell einen wichtigen Überlebensfaktor für Tumorzellen dar. Interessanterweise exprimieren epidermale Zellen von K5-SOS transgenen Mäusen große Mengen an Vaskular-Endothelialem Wachstumsfaktor (VEGF) und β1 Integrin, welche bedeutende Regulatoren der Tumorangiogenese bzw. Tumorzellmigration sind. Um die Mechanismen zu untersuchen, wie die Signale ausgehend von EGFR, VEGF oder β1 Integrin zur Tumorbildung und zum Tumorwachstum führen, wurden Mäuse generiert, in denen VEGF oder β1 Integrin spezifisch in der Epidermis deletiert werden können ($VEGF^{\Delta ep}$ und $\beta1int^{\Delta ep}$), und mit K5-SOS Mäusen gekreuzt. K5-SOS transgene epidermale Zellen besitzen ein verstärktes Migrationspotenzial sowie weniger fokale Kontakte, was durch den Src-Kinase Inhibitor SU6656 aufgehoben werden kann. Interessanterweise entwickeln K5-SOS transgene Mäuse ohne β1 Integrin Expression in der Epidermis im Gegensatz zu Kontrollmäusen keine Tumore, und diese Tatsache konnte in Zusammenhang mit Signal-Effektoren von β1 Integrin und EGFR gebracht werden. Die Expression von K5-SOS in den Hautzellen von $\beta1int^{\Delta ep}$ Mäusen hat den Phänotyp dieser Mäuse dramatisch verschlechtert, was sich hauptsächlich durch eine verdickte, hyperproliferative Epidermis und degenerierende Haarfollikel zeigte. Weiters hat eine Epidermis-spezifische Deletion von β1 Integrin in Mäusen mit bereits bestehenden Tumoren das Tumorwachstum signifikant verzögert. Diese Ergebnisse demonstrieren eine entscheidende Funktion von β1 Integrin in der Krebsentstehung, Tumorhomeostase und –wachstum, und belegen eine Wechselwirkung zwischen EGFR und β1 Integrin *in vivo*.

Die Epidermis-spezifische Deletion von VEGF hat ebenfalls das K5-SOS-abhängige Hauttumorwachstum beeinträchtigt. Überraschenderweise hat das

Fehlen von VEGF in einem EGFR-mutanten (waved-2; wa2) Hintergrund die Tumorentwicklung komplett inhibiert, was darauf hinweist, dass VEGF und EGFR in neoplastischen Zellen zusammenarbeiten, um das Tumorwachstum zu fördern. Mechanistisch konnte ich zeigen, dass K5-SOS die Expression von VEGF und seinen Rezeptoren Flt1 und Neuropilin-1 in einer Erk-abhängigen Weise hochreguliert, wobei ein autokriner Proliferationskreis aktiviert wird. Währenddessen fungiert der EGFR als Überlebensfaktor für Tumorzellen. Darüber hinaus konnte ich zeigen, dass Flt1 in einer Vielzahl von humanen Plattenepithelkarzinomen exprimiert ist, und dass die Inhibierung von Flt1 in Plattenepithelkarzinom-Zelllinien deren Proliferation beeinträchtigt, was die medizinische Relevanz meiner Ergebnisse unterstreicht. Somit sollte VEGF neben seiner regulatorischen Funktion in der Angiogenese als sehr potenter Wachstumsfaktor für epidermale Tumore angesehen werden.

Zusammenfassend demonstrieren meine Studien, dass der EGFR essentiell für die Hautentwicklung während der Embryogenese und der ersten Zeit nach der Geburt ist, um ein späteres Überleben zu gewährleisten. Weiters hat der EGFR eine Schlüsselfunktion in der Regulation des Haarfollikelzyklus und in den komplexen Prozessen der Wundheilung. Außerdem spielt der EGFR eine entscheidende Rolle in der Entstehung und dem Wachstum epithelialer Tumore. Im Wesentlichen demonstriert diese Studie zum ersten Mal, dass EGFR und VEGF in Tumorzellen kooperieren, und bietet eine molekulare Erklärung dafür, warum kombinierte anti-EGFR und anti-VEGFR Therapien viel effizienter als Einzeltherapien in der Behandlung von humanen Tumoren sind.

2 SUMMARY

Epidermal growth factor receptor (EGFR) deficient mice die at different stages of embryonic and early postnatal development and develop epithelial phenotypes and a neurodegenerative disease. Besides controlling the normal development of astrocytes and epithelial cells, EGFR overexpression has been detected in many human carcinomas and glioblastomas, which are tumors of epithelial and glial origin, respectively. Transgenic mice expressing an activated form of the Ras activator Son of Sevenless from the epithelial-specific keratin 5 promoter (K5-SOS) develop skin tumors in an EGFR-dependent manner. In these tumors the EGFR provides an essential survival signal to tumor cells most likely by activating Akt. The study of the physiological signaling pathways of the EGFR in epithelial cells is therefore of fundamental importance for understanding how aberrant EGFR signaling can lead to tumor formation and progression.

Due to the early postnatal lethality of EGFR-null mice the role of EGFR signaling during skin and hair follicle development could not be studied. Therefore, mice carrying conditional EGFR (floxed) alleles were crossed with the K5-Cre or the tamoxifen-inducible K5-CreER^{T} transgenic lines to generate mice which would allow EGFR deletion specifically in the basal layers of the epidermis both during embryogenesis (EGFR$^{\Delta ep}$) and in adult mice (EGFR$^{\Delta epER}$), respectively. Surprisingly, epidermis-specific EGFR inactivation during embryogenesis or within the first postnatal days resulted in early postnatal lethality. In contrast, EGFR deletion in the skin at later time points led only to mild derangements of hair follicles and mice were viable. The formation of the pivotal skin barrier was delayed in EGFR deficient as well as EGFR$^{\Delta ep}$ mice, but became impermeable shortly after birth.

Moreover, I could demonstrate that the lack of EGFR in the epidermis and the outer root sheath of hair follicles delayed both hair follicle morphogenesis and the entry into the hair growth cycle. In addition, EGFR signaling is involved in the inflammatory response of the skin, as Langerhans cells, αβT-cells, DCs, granulocytes and mast cells infiltrate independently of the presence of hair follicles. Importantly, my studies also revealed that in the absence of EGFR

wound healing of full-thickness punch wounds is delayed by affecting reepithelialization and wound contraction.

Besides its crucial role in the development and maintenance of the skin and its appendages, the EGFR has been shown to be implicated in skin tumorigenesis. As mentioned previously, in a K5-SOS-dependent tumor model EGFR was identified as a survival factor for tumor cells. Interestingly, epidermal cells isolated from K5-SOS transgenic mice expressed high levels of the vascular endothelial growth factor (VEGF) and β1 integrin, which are major regulators of tumor angiogenesis and tumor cell migration, respectively. To address the mechanisms by which signaling events downstream of EGFR and/or VEGF or β1 integrin lead to tumor formation and progression, mice in which VEGF or β1 integrin could specifically be deleted in the epidermis ($VEGF^{\Delta ep}$ and $\beta1int^{\Delta ep}$) were generated and crossed to K5-SOS mice. K5-SOS transgenic epidermal cells exhibited an enhanced migratory potential as well as reduced numbers of focal contacts, which could be reversed by the Src kinase-specific inhibitor SU6656. Interestingly, K5-SOS transgenic mice lacking β1 integrin in the epidermis did not develop any tumors when compared to littermate controls, and this phenotype could also be related to downstream signaling effectors of β1 integrin and the EGFR. Expression of K5-SOS in epidermal cells of $\beta1int^{\Delta ep}$ mice dramatically exacerbated the phenotype of these mice, which is mainly characterized by a thickened and hyperproliferative epidermis and regressing hair follicles. Furthermore, epidermis-specific deletion of β1 integrin in mice with already existing tumors significantly delayed tumor growth. These findings demonstrate a pivotal role for β1 integrin in tumor initiation as well as tumor maintenance and progression, and provide evidence for a crosstalk between the EGFR and β1 integrin *in vivo*.

Epidermis-specific deletion of VEGF also delayed K5-SOS-dependent skin tumor growth. Surprisingly, complete inhibition of tumor development was found in the absence of VEGF in a mutant EGFR (waved-2; wa2) background, demonstrating that VEGFR and EGFR signaling synergize in neoplastic cells to promote tumor growth. Similar results were obtained with therapeutics inhibiting EGFR and VEGFR. Mechanistically, I could show that K5-SOS upregulates VEGF and its receptors Flt1 and Neuropilin-1 in an Erk-dependent manner thereby activating an autocrine proliferation loop, whereas EGFR acts as a

survival factor for tumor cells. Furthermore, Flt1 was found to be expressed in the majority of human squamous cell carcinomas (SCC) and its inhibition in human SCC cell lines impairs proliferation emphasizing the medical relevance of these findings. Thus, in addition to regulating angiogenesis, VEGF has to be considered as a potent growth factor for epidermal tumors.

Taken together, my studies demonstrate that EGFR is indispensable for the development of the skin during embryogenesis and early postnatal life to guarantee later survival, and that it is a key player in regulating the hair cycle clock and the complex processes of wound repair. Moreover, EGFR plays a major role in the initiation and progression of epithelial cancer development. Importantly, this study represents the first demonstration of a synergistic action of EGFR and VEGF signaling in tumor cells and provides a molecular explanation why combined anti-EGFR and anti-VEGFR therapies might be more efficient than single therapies for the treatment of human cancer.

3 INTRODUCTION

3.1 The skin and its appendages

3.1.1 Skin development

The skin is the interface of the body with the environment and it serves as a mechanical, chemical and immunological protective barrier against external environmental insults and loss of essential body fluids. To cope with the daily attacks of wounds, scratches, chemical carcinogens and UV light the skin epidermis undergoes continual self-renewal to repair injured tissue and replace old cells. The maintenance of tissue homeostasis depends on stem cells residing in the epidermis, the adult hair follicle and sebaceous gland (Fuchs, 2007; Koster and Roop, 2007).

The epidermis develops from a single layer of ectodermal cells. The majority of the cells in the basal layer are rapidly dividing progeny of stem cells, the transit-amplifying cells, which undergo a limited number of divisions before they descend to terminal differentiation (Fuchs and Horsley, 2008). Epidermal differentiation results in an organized tissue in which morphologically distinguishable cells are arranged in discrete layers of basal, spinous, granular, and cornified cells. The first layer, the stratum basale, is attached to the dermo-epidermal junction (basement membrane, BM) - which is rich in extracellular matrix and growth factors and provides proliferative stimuli to the basal layer of the epidermis - and by dividing supplies successors for the cells lost at the skin surface. Once cells leave the basal layer to enter the stratum spinosum, they loose the capacity to divide, they increase in size, flatten, and their water content diminishes. In parallel, proteins that are no longer used, nucleic acids, mitochondria and plasma membranes are successively destroyed. The ultimate product of keratinocyte differentiation, the corneocyte, consists essentially of the cornified envelope filled with keratin bundles and provides a mechanically resistant cell surface (Fig.1) (Fuchs, 1990, 2007; Reichert, 1993).

Besides this remarkable proliferative potential, the epidermis is able to generate different appendages: hair follicles (HF) and their associated sebaceous glands (SG) as well as sweat glands in nonhaired regions (Fuchs and Horsley, 2008).

3.1.2 Hair follicle morphogenesis

The hair follicle is a unique characteristic of mammals and the only organ that throughout life undergoes cyclic transformations. In mice, HF morphogenesis occurs in waves from embryonic day 12.5 to 18.5 and results in a number of different types of hair: primary or tylotrich (guard) HF, characterized by a large hair bulb, long straight hair, and two sebaceous glands, secondary or non-tylotrich (awl, auchene, and zigzag) HFs with thinner and shorter hair shafts and one sebaceous gland, and Vibrissa HFs which have specialized sensory functions (Schmidt-Ullrich and Paus, 2005).

HF morphogenesis starts with the condensation of specialized dermal cells beneath the epidermal layer which induce the formation of a focal thickening in the basal layer of the epidermis (hair germ or hair placode), and stimulate epidermal stem cells to grow downward and invaginate into the dermis. Thereby, the so called dermal papilla (DP) is formed, which drives further HF formation. As the follicle grows down it is encapsuled by the highly proliferative matrix cells at the leading edge. The inner layers of the HF differentiate into concentric cylinders and generate the inner root sheath (IRS) and the central hair shaft (HS). Once the HF reaches the bottom of the dermis, the HF becomes fully mature.

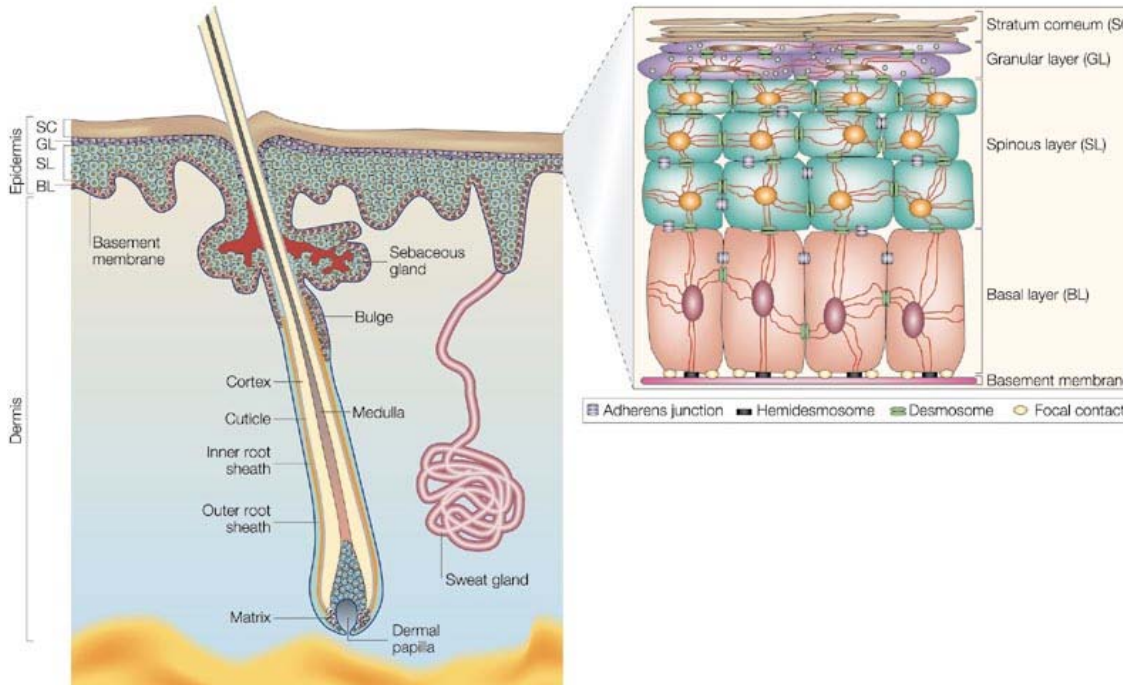

Figure 1. The skin and its appendages. Cross-section through mammalian skin and a hair follicle (Fuchs and Raghavan, 2002).

However, the matrix cells continue dividing and their successors terminally differentiate to form the growing hair that exits the skin surface. In mouse back skin HF morphogenesis is completed between postnatal day 6 and 8 (Botchkarev and Paus, 2003; Fuchs and Horsley, 2008; Schmidt-Ullrich and Paus, 2005).

3.1.3 Hair follicle cycle

Matrix cells are transit amplifying cells which undergo only a limited number of divisions before they differentiate. Therefore at some point of hair growth the supply of matrix cells declines. This is when HFs enter the so called HF cycle which starts with a degenerative phase called catagen. In mice, catagen occurs in waves starting from the head caudally towards the tail and laterally down the sides of the mouse. This usually happens between postnatal day 14 and 18 and lasts 3 to 4 days (Alonso and Fuchs, 2006).

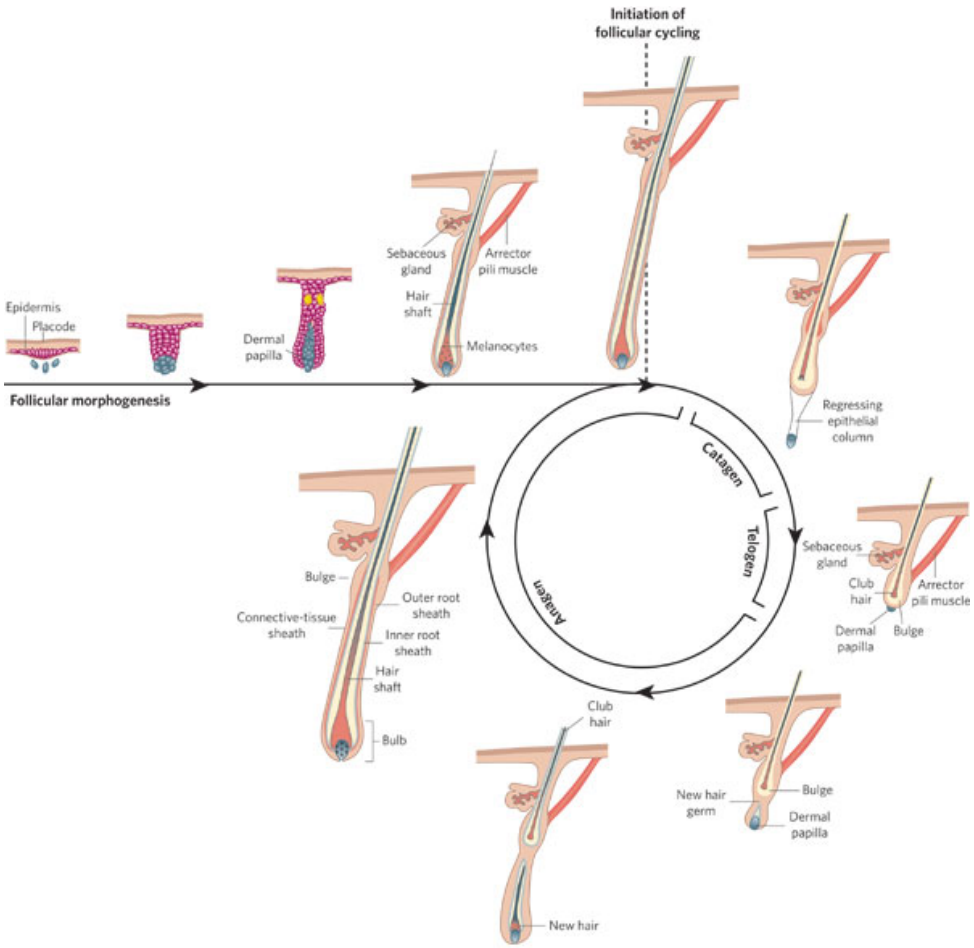

Figure 2. The hair cycle. Hair follicles undergo cyclic transformations from stages of rapid growth (anagen) to apoptosis-driven regression (catagen) and back to anagen, via an interspersed period of relative quiescence (telogen) (Fuchs, 2007).

During catagen apoptosis of epithelial cells in the bulb and outer root sheath leads to a complete regression of the lower “cycling” portion of the HF (Fig. 2). This translocates the dermal papilla upward to rest below the permanent, non-cycling upper follicle.

After this destructive phase HFs enter a quiescent, resting stage called telogen, which lasts only 2 days in the first and more than 2 weeks in the second hair cycle. Once quiescent epidermal stem cells located in the lowest permanent portion of the HF, the bulge region (Fig. 1), are activated, they become transit-amplifying cells and divide rapidly to regenerate the lower portion of the hair follicle in a phase called anagen which resembles HF morphogenesis (Fig. 2) (Alonso and Fuchs, 2006; Fuchs, 2007).

The terminally differentiated cells of the sebaceous gland are sebocytes filled with lipids which burst and release their contents to lubricate the skin surface.

In order to maintain skin homeostasis and to control hair follicle growth and regression, proliferation and differentiation of epidermal cells must be tightly regulated and coordinated. Too little proliferation leads to thinning of the skin and to loss of the skin’s barrier function, whereas too much proliferation may result in hyperproliferative diseases such as psoriasis or cancer (Fuchs, 2007; Lowes et al., 2007). We still know very little about the signaling pathways that are involved in skin development and the mechanisms that actively orchestrate epidermal cell fate. Studies over the past ten years have implicated several signaling pathways, including the Wnt, FGF, Notch, Bmp, SHH and TGFβ pathways in the development and homeostasis of the skin and its appendages.

3.2 Wound repair

Injury to the skin excites a complex cascade of events involving inflammation, new tissue formation and remodeling of new tissue, which ultimately results in at least partial reconstitution of the wounded skin (Schafer and Werner, 2007; Werner and Grose, 2003).

Immediately after wounding the repair process is initiated by the release of soluble mediators such as growth factors, cytokines and low-molecular-weight compounds from degranulating platelets and from the serum of damaged blood vessels (Werner and Grose, 2003). Furthermore the injury of blood vessels

leads to the formation of the blood clot which serves as a first barrier against invading microorganisms, as a scaffold for invading cells and as a reservoir of growth factors essential in later stages of the repair process. Neutrophiles invade the wound within minutes after injury, and are accompanied by lymphocytes and monocytes, which differentiate into macrophages, within the next 2 or 3 days. These cells are involved in the clearance of microorganisms and phagocytosis of cell debris. Moreover, they release a multitude of cytokines which boost proliferation, migration, and survival of various cell types at the site of injury (Fig. 3A) (Werner and Grose, 2003). The second stage of wound repair, new tissue formation, is characterized by the migration of keratinocytes of the damaged epidermis and HFs over the injured dermis, followed by keratinocyte proliferation at the wound edge (Fig. 3B). After this process of reepithelialization keratinocytes differentiate to restore the barrier function. In addition, fibroblasts attracted from the wound edge or the bone marrow migrate and proliferate in order to repair the injured dermis. Some fibroblasts differentiate into myofibroblasts, contractile cells responsible for wound contraction. New blood vessels form and nerve sprouting occurs at the wound edge thereby replacing the fibrin matrix by the so called granulation tissue (Gurtner et al., 2008; Schafer and Werner, 2007). 2 to 3 weeks after injury the third stage of wound repair – remodeling – begins. This phase is characterized by synthesis and remodeling of the collagen matrix which is mainly achieved by matrix metalloproteinases sectreted by fibroblasts, endothelial cells, and macrophages.

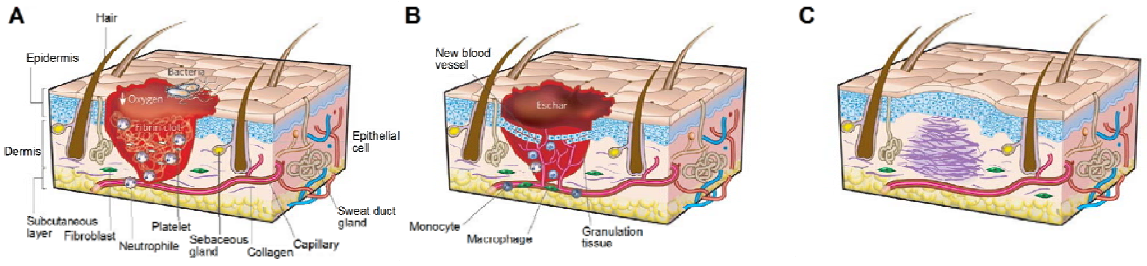

Figure 3. Classical stages of wound repair. Injury to the skin initiates a complex cascade of events involving inflammation **(A)**, new tissue formation **(B)** and remodeling **(C)** (Gurtner et al., 2008).

Finally, the processes initiated by the injury need to be terminated, and the majority of endothelial cells, macrophages, and myofibroblasts undergo

apoptosis or exit from the wound. The resulting scar lacks hair follicles, sebaceous and sweat glands and is mechanically insufficient and the tissue never retrieves the properties of healthy skin (Fig. 3C) (Gurtner et al., 2008; Schafer and Werner, 2007). Scarring can also be excessive and eventually leads to hypertrophic scars and keloids.

Interestingly, in mammalian embryos wound healing results in a perfect repair without scarring, which suggests fundamental differences in the wound repair process between embryonic and adult mammals (Mackool et al., 1998; Martin, 1997).

3.3 Epidermal cancers

The skin is subject to sustained environmental assaults. As a result, epidermal cells have a high risk of acquiring oncogenic mutations. However, proportionally few skin cancers develop since one mutation is not enough to cause cancer and the majority of cells with oncogenic mutations are lost through the natural process of terminal differentiation. Tumors usually arise clonally and an estimated number of 2 to 3 genetic lesions in rodents and 5 events in humans is essential to transform a cell (Owens and Watt, 2003). Thus, preferably stem cells, the long-term residents of the skin, are capable of accumulating enough oncogenic events to induce tumor growth. Nevertheless, even though stem cells are the primary target for the accumulation of oncogenic alterations, the differentiation fraction may also contribute to tumor formation. Besides the fact that transit-amplifying cells and also post-mitotic, terminally differentiating epidermal cells can proliferate extensively as a result of oncogenic transformation (Pelengaris et al., 1999), differentiated cells may influence the tumorigenic potential of a mutated stem cell by enhancing or inhibiting its clonal expansion for example by releasing growth factors or by changing the expression of cell adhesion molecules thereby affecting direct cell-cell contacts (Owens and Watt, 2003).

Epidermal cancer comprises a variety of different tumor types, including basal cell carcinoma (BCC), squamous cell carcinoma (SCC), trichofolliculoma, pilomatricoma as well as sebaceous adenoma, with BCC and SCC (papilloma and SCC in mice) being the most common epithelial tumors of the skin in

humans. The diversity of epidermal cancers reflects the repertoire of differentiated cell types in healthy epidermis. Thus, the kind of tumor that arises essentially depends on the nature of the oncogenic alteration and the type of cell that acquired them (Owens and Watt, 2003). Many of the genes affected in epidermal cancers have been identified, such as p53, factors belonging to the WNT, SHH or Ras signaling pathways, just to mention a few of them.
Substantial research is being done in order to find new targets for cancer therapy and to develop new and more effective drugs.

3.4 Growth factors controlling skin physiology and pathology

The development and homeostasis of the skin essentially depends on a variety of growth factors. By binding to the respective receptors on the cell surface, growth factors transduce extracellular signals through a complex network of signaling cascades (or directly through receptor translocation) to the nucleus, thereby controlling proliferation, differentiation, survival, migration or the fate of the respective cell. Besides, growth factors are necessary for cell-cell communication, which is absolutely required to accomplish the well orchestrated processes responsible for the development of organized tissues. Therefore, it stands to reason that upon injury secretion of different growth factors is accelerated in a well established sequence in order to initiate and succeed wound repair. Moreover, it is not surprising that tumors seize the same growth factors and signaling pathways that are implicated in wound repair to grow, to survive and to migrate. What was suggested already thirty years ago (Dvorak, 1986) was confirmed by DNA-microarrays only recently: highly malignant tumors and the tissue of healing skin wounds display a similar gene expression pattern (Cole et al., 2001; Cooper et al., 2005). Therefore, tumors are also called “wounds that do not heal” (Dvorak, 1986). Growth factors involved in both wound healing and skin cancer belong to different families: the platelet-derived growth factor (PDGF) family, fibroblast growth factor (FGF) family, epidermal growth factor (EGF) family, insulin-like growth factor (IGF) family, Transfoming growth factor β (TGFβ) family, angiopoietins and the vascular endothelial growth factor (VEGF) family. While the VEGF family does not have a profound impact on skin development, it was shown that epidermal-

specific deletion of VEGF delays wound healing and inhibits tumor formation due to impaired wound and tumor angiogenesis (Rossiter et al., 2004).

Of the many growth factor receptor tyrosine kinases (RTK), the EGF family of RTKs is probably one of the most extensively studied for its function in skin development, physiology, and cancer. As described in detail below EGFR deficient mice display several skin and hair growth defects, EGFR has been implicated in different processes of wound repair, and it was shown to be involved in the formation and progression of many murine and human tumors of epithelial origin (Schneider et al., 2008b; Sibilia et al., 2007).

Two decades of research have revealed that all stages of wound healing are controlled by a plethora of different growth factors and cytokines. For example, reepithelialization was shown to be positively regulated by members of the FGF, the EGF and the PDGF family, and by the hepatocyte growth factor (HGF). In contrast, TGFβ negatively regulates wound reepithelialization. The VEGF family of growth factors and angiopoietins are important for wound angiogenesis. In addition to growth factors several cytokines including MCP-1/CCL2, MIP-1α, GM-CSF or interleukins were identified as important players in wound repair (Gurtner et al., 2008; Werner and Grose, 2003). More recently transcription factors that modulate gene expression at the wound site have been brought into focus of researchers. AP-1, PPARβ/δ, c-Myc, Egr-1, E2F-1, HoxA3, and HoxD3, Smad2, Smad3, and Stat3 have been identified to regulate reepithelialization, whereas Grainy head transcription factors were shown to be involved in controlling the subsequent reestablishment of the epidermal barrier function. Inflammation at the wound site is modulated by Nrf2, Smad3 and nuclear receptors such as glucocorticoid, estrogen, androgen, and the PPARα receptors. Angiogenesis of the wound is induced by HoxA3, HoxD3, Egr-1, and CARP. Moreover, VEGF expression is controlled by HIF-1α and Sp1. Whereas glucocorticoids and HoxB13 reduce fibroplasia and scarring, Smad3, c-Myb, and β-catenin facilitate these processes (Schafer and Werner, 2007).

3.4.1 The Epidermal growth factor receptor (EGFR)

3.4.1.1 EGFR signaling

The epidermal growth factor receptor (EGFR), also known as ErbB1/HER1, is a member of a family of structurally related tyrosine kinase receptors that includes ErbB2/HER2/Neu, ErbB3/HER3, and ErbB4/HER4 (Schlessinger, 2002). All four receptors have a common structure comprising an extracellular ligand-binding domain, a single hydrophobic membrane-spanning region and a cytoplasmic tyrosine kinase domain flanked by a carboxy-terminal tail with tyrosine autophosphorylation sites (Citri and Yarden, 2006; Yarden and Sliwkowski, 2001). While EGFR and ErbB4 are functional autonomously, the other two receptors, ErbB2 and ErbB3 are non-autonomous as ErbB2 can not bind any ligands and ErbB3 is defective in its tyrosine kinase activity (Bublil and Yarden, 2007; Citri et al., 2003; Guy et al., 1994).

To date seven ligands capable of binding EGFR have been identified: amphiregulin (AR), betacellulin (BTC), heparin-binding EGF-like growth factor (HB-EGF), transforming growth factor α (TGFα), epiregulin (EREG), epigen (EPGN), and epidermal growth factor (EGF) itself. A genome-wide screen using algorithms based on genomic and cDNA structures revealed that additional potential EGFR ligands are unlikely (Kochupurakkal et al., 2005). While EGF, TGFα, AR and EPGN bind exclusively to EGFR, HB-EGF, BTC and EREG can bind and activate ErbB4 as well (Fig. 4) (Beerli and Hynes, 1996; Harris et al., 2003).

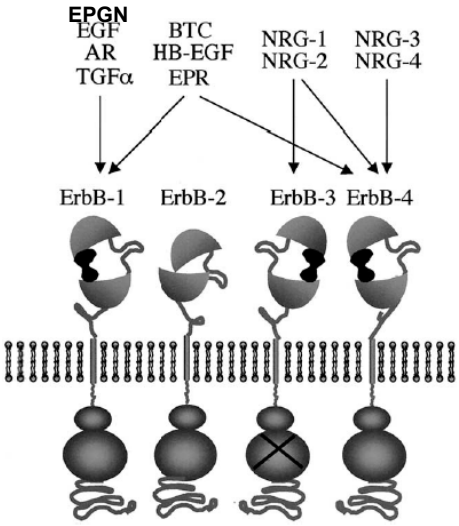

Figure 4. ErbB receptors and their ligands. ErbB receptors are depicted as multilobular transmembrane structures. The highly homologous cytoplasmic regions contain a bilobular tyrosine kinase domain, flanked by a short transmembrane stretch and a long autophosphorylation tail. Note that the kinase domain of ErbB3 is catalytically inactive. The extracellular domains comprise two cysteine-rich domains (represented by loops), which mediate ligand-induced dimerisation. Eleven growth factors and their ErbB specificities are depicted. Adapted from (Marmor et al., 2004).

EGFR ligands share a conserved EGF motif that is flanked by an N-terminal extension and a C-terminal anchoring region, which keeps the ligand precursors attached to the cell membrane. The ligands are initially synthesized as transmembrane precursors which are subsequently cleaved (shedded) by cell surface proteases to release mature, soluble growth factors (Harris et al., 2003). Zinc-dependent membrane associated proteases called ADAM proteases (a disintegrin and metalloproteases) have been implicated in the shedding of most of the EGFR ligands (Blobel, 2005). Released ligands activate EGFR in an autocrine, paracrine or endocrine manner, that is on the cell of its origin, on neighboring cells or after systemic distribution on distant cells. Moreover, precursors of TGFα, HB-EGF and AR have been shown to act also in a juxtacrine mode by stimulating adjacent cells via cell-cell contacts, which likely results in distinct biological responses as compared with the signaling transduction induced by soluble growth factors (Singh and Harris, 2005).

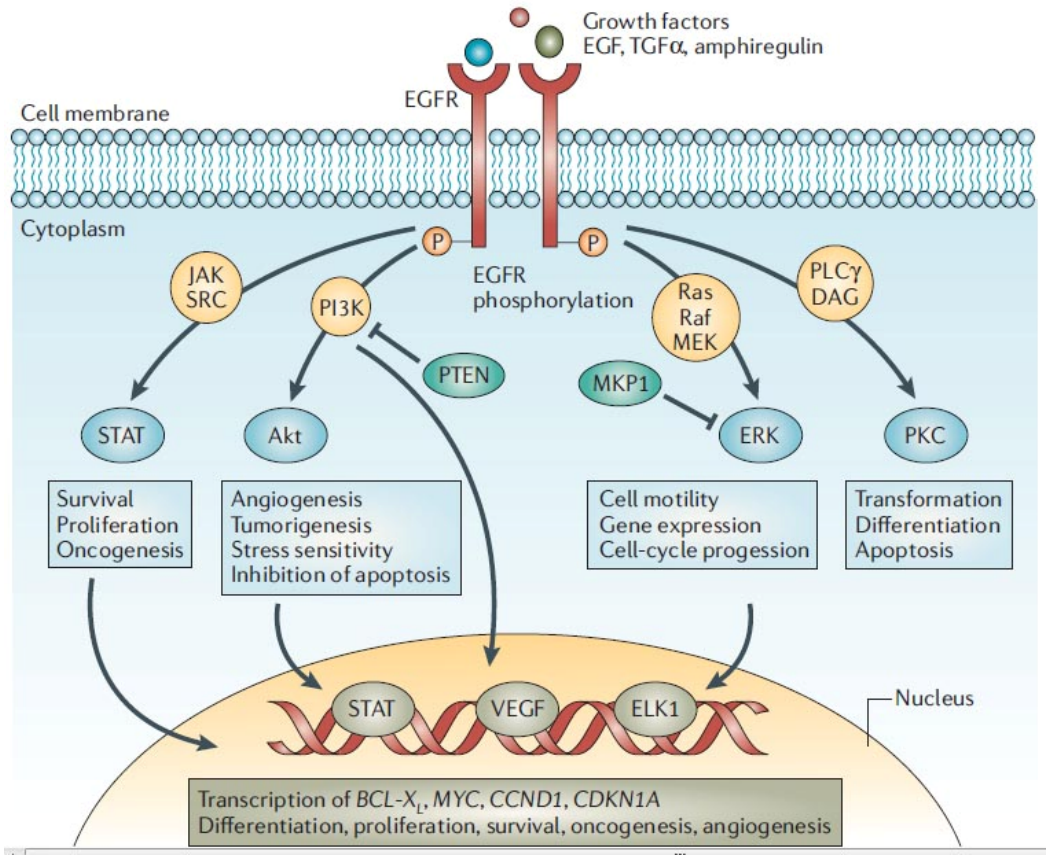

Figure 5. The main downstream signaling pathways regulated by EGFR. Adapted from (Nyati et al., 2006)

Ligand binding induces receptor dimerization and activation of the intrinsic tyrosine kinase with subsequent autophosphorylation of key tyrosines located within the carboxy-terminal tail of the receptor (Schlessinger, 2002; Yarden and Sliwkowski, 2001). Phosphorylated tyrosine residues enable the recruitment and activation of proteins containing phosphotyrosine binding domains and Src-homology 2 domains (SH2) such as Grb2, SHC and PLCγ which in turn activate complex downstream signal transduction pathways, thus transducing extracellular stimuli to the nucleus (Fig. 5) (Hynes and Lane, 2005; Schlessinger, 2002; Yarden and Sliwkowski, 2001).

The identity and relative strength of the cellular response is thought to be determined by the ligand and the nature of the various signaling molecules recruited to the different sites of autophosphorylation of the receptor (Fig. 6). Furthermore, dimerisation of ErbBs can take place between two identical receptors (homodimerisation) or with any of the three other members of the ErbB family (heterodimerisation) depending on which receptor proteins are expressed in a given cell. This increases the number of signaling pathways that can be activated after EGFR stimulation thereby augmenting the signaling complexity needed to govern cell proliferation, differentiation, migration, and survival (Hynes and Lane, 2005; Schlessinger, 2002; Yarden and Sliwkowski, 2001).

The major pathways activated upon EGFR stimulation are the Ras-Raf-MEK-ERK1/2, signal transducer and activator of transcription 1 (STAT1), STAT3 and STAT5 pathways controlling proliferation and differentiation, and the pro-survival and anti-apoptotic PI3K-Akt-mTOR pathway (Fig. 5). In addition to ligand-mediated EGFR induction, the EGFR can also be transactivated by a multitude of G-protein-coupled receptors, integrins, and cytokine receptors (Cabodi et al., 2004; Hynes and Lane, 2005; Schlessinger, 2002; Yarden and Sliwkowski, 2001).

Signal attenuation is predominantely achieved by the internalization of receptor-ligand complexes through clathrin-coated invaginations of the cell membrane. Distinct sorting pathways either redirect the receptor back to the cell surface (receptor recycling) or upon ubiquitination to lysosomes for degradation (Husnjak and Dikic, 2006; Waterman and Yarden, 2001). In addition, a variety of suppressive mechanisms attenuates ligand-induced signaling, for example by

ligand depletion, dephosphorylation and kinase inactivation. Moreover, signal desensitization is induced by a plethora of transcriptional repressors and RNA-binding proteins such as LRIG, ARGOS or RALT (Shilo, 2005).

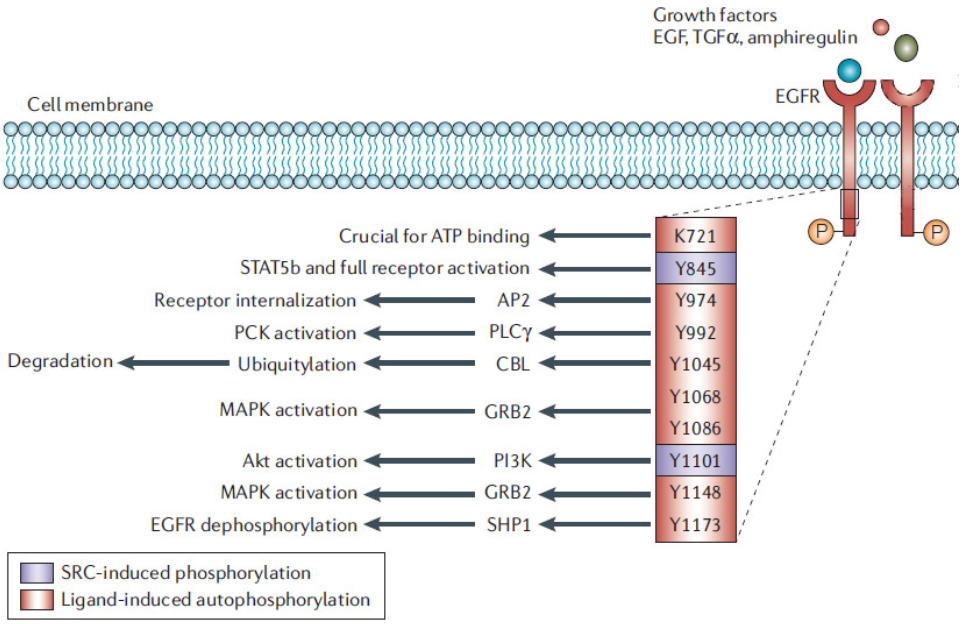

Figure 6. The main downstream signaling pathways regulated by EGFR. Ligand binding can induce homo- or heterodimerization, which subsequently activates many sites within the C terminus such as Y992, Y1045, Y1068, Y1086, Y1148 and Y1173 (shown in red). Elsewise SRC non-receptor kinase can phosphorylate Y845 and Y1101 (shown in purple). Adapted from Nyati et al., 2006.

MAPK pathway

One of the best characterized pathways downstream of the EGFR is the MAPK pathway (Fig. 5). Upon ligand-induced receptor dimerization and activation, the adapter protein Grb2 binds to phosphorylated tyrosines at the C-terminus of the receptor and interacts via its SH3 domain with the nucleotide exchange factor Son of Sevenless (SOS), thereby recruiting SOS to the plasma membrane (Schlessinger, 1994; Weiss et al., 1997). SOS catalyses the activation of Ras by facilitating GDP-GTP exchange. Activated, GTP-bound Ras binds to the cytoplasmic serine/threonine protein kinase Raf, which leads to its translocation to the plasma membrane and subsequently to its activation (Marais et al, 1995). Raf phosphorylates the MAPK/ERK kinase (MEK) proteins, which in turn activate the extracellular signal-regulated kinase (ERK) subgroup of mitogen-activated protein (MAP) kinases. In the activated state ERKs translocate to the nucleus and induce expression of immediate early genes, such as *c-fos*. Transcriptional activation of AP-1 target genes by ERK is mediated via

phosphorylation and subsequent activation of TCF/Elk-1 (Schlessinger and Ullrich, 1992).

PI3K/Akt pathway

The phosphatidylinositol 3-kinase (PI3K)/Akt is another key signaling pathway activated by EGFR. However, EGFR lacks the motifs that allow direct binding to the p85 regulatory subunit of PI3K. These motifs are present in RTKs like ErbB3 (Prigent and Gullick, 1994), and PDGF or in docking proteins such as IRS1 (Insulin receptor substrate 1) and Gab1 (Lehr et al., 1999). Therefore, PI3K activation by EGFR most likely happens indirectly by heterodimerizing with ErbB3 or via adaptor proteins such as Gab1 (Prigent and Gullick, 1994; Soltoff et al., 1994). Phosphorylated, activated PI3K mediates the formation of phosphatidylinositol-3,4,5-triphosphate (PIP_3), which transduce signals from the cell surface to the cytoplasm. PIP_3 activates the 3-phosphoinositide-dependent protein kinase-1 (PDK1), which in turn activates the serine/threonine kinase Akt, also known as protein kinase B (PKB) (Citri et al., 2003; Dillon et al., 2007).

Activated Akt has several effects, both in the cytoplasm and the nucleus. It promotes cell survival and blocks apoptosis by a variety of routes. For instance, Akt-mediated phosphorylation of BCL2 antagonist of cell death (Bad) blocks apoptotic activity to promote cell survival. Furthermore, phosphorylation of pro-caspase 9 or the Forkhead (FKHR) family of transcription factors (FOXO) by Akt inhibit the induction of apoptosis by these factors (Dillon et al., 2007). In addition, phosphorylation of Ikappa-B kinase by Akt leads to activation of the transcription factor NF-kB that results in the expression of several prosurvival genes (Dillon et al., 2007). Moreover, Akt-mediated activation of mammalian target of rapamycin (mTOR) was shown to be important for stimulating cell proliferation. Importantly, the PI3K/Akt pathway also induces the expression of angiogenic factors such as VEGF or hypoxia inducible factor-1α(HIF1α) (Hennessy et al., 2005).

STAT pathway

Phosphorylated EGFR can also - directly or indirectly - activate signal transducer and activator of transcription 1 (STAT1), STAT3 and STAT5. Upon phosphorylation the activated STAT transcription factors translocate into the

nucleus and directly regulate gene expression crucial for cell survival, proliferation, transformation and oncogenesis (Bowman et al., 2000).

Transactivation of EGFR

As mentioned previously, in addition to ligand-mediated EGFR induction, the EGFR can also be transactivated by a multitude of G-protein-coupled receptors, integrins, and cytokine receptors (Cabodi et al., 2004; Hynes and Lane, 2005; Schlessinger, 2002; Yarden and Sliwkowski, 2001).

Integrins are adhesive receptors, which are composed of α- and β-subunits, and are essential for the anchorage of extracellular matrix proteins to the actin cytoskeleton, thereby mediating adhesion and migration of epidermal cells (Brakebusch and Fassler, 2005). Integrins transduce signals in a bidirectional manner across the plasma membrane. Intracellular signals result in conformational changes in the integrin ectodomain leading to a ligand-competent state of the receptors. Binding of extracellular ligands mediates, in turn, structural changes that transduce distinct signals inside the cell. They trigger a multitude of signaling pathways which affect cell migration, proliferation, differentiation, and survival, and the outcome of the signal depends on the differential expression of more than 20 different subunits of integrins and the specific localization of the receptors (Hynes, 2002). Due to the lack of an actin binding domain and enzymatic activity within the integrin molecules, integrin associated proteins such as α-actinin, talin, filamin, and integrin linked kinase (ILK), Rack1 or caveolin are required for the signal transduction (Brakebusch and Fassler, 2005). It has been demonstrated that integrins interact with various signal-transducing components of focal adhesions, particularly the focal adhesion kinase (FAK) and c-Src. FAK has been shown to bind the cytoplasmic tail of β1 integrin via its amino-terminal domain, and to bind the SH2 and SH3 domains of other focal adhesion proteins via its carboxy-terminal domain. Upon integrin-mediated activation FAK undergoes autophosphorylation, thereby creating a high affinity binding site for the SH2 domain of Src, which in turn triggers transphosphorylation of FAK at different tyrosine residues, rendering FAK a fully active kinase. Phosphorylated FAK can form a complex with Grb2 and SOS, and, thus, activates the MAPK pathway (Mitra et al., 2005; Schlaepfer and Hunter, 1997).

Many integrin-mediated signaling pathways are very similar to those triggered by growth factor receptors and are closely connected to them. In fact, it was demonstrated that many cellular responses to soluble growth factors, such as EGF or PDGF, essentially depend on the adherence of cells to a substrate via integrins (Hynes, 2002). It was shown that integrins and growth factor receptors form complexes at the cell membrane, and that integrin-dependent adhesion triggers ligand-independent activation of the EGFR signaling (Bill et al., 2004; Cabodi et al., 2004).

Interestingly, mice lacking expression of β1 integrin in the skin have a similar phenotype like EGFR deficient mice (Brakebusch et al., 2000; Raghavan et al., 2000). They display several epithelial defects including the failure to develop a normal hairy coat or they turn bald with time. Moreover, defects in wound healing have been demonstrated for these mutants, and β1 integrin has been implicated in the formation and progression of epithelial tumors (Brakebusch and Fassler, 2005; Grose et al., 2002). Thus, integrin and EGFR signaling might act on parallel signal transduction cascades and might need to synergize to reach a full biological response.

3.4.1.2 EGFR in skin development

A large body of evidence indicates that EGFR plays an important role in regulating the development of the epidermis and its appendages. In the skin, EGFR expression is most abundant in the basal layer of the epidermis and in the outer root sheath of the hair follicles, where the proliferating cells reside. As soon as keratinocytes withdraw from the cell cycle, differentiate and migrate to the suprabasal epidermal layers EGFR expression is downregulated (Sibilia and Wagner, 1995). A multitude of naturally occurring and experimentally induced mutant mice reveal that EGFR signaling has an impact on the physiological development of epithelia. Mice homozygous for a disrupted TGFα gene display severe derangements of hair follicles, resulting in a wavy coat and curly whiskers (Mann et al., 1993). The naturally occurring mouse mutant strains waved-1 and waved-2, which carry null mutations in the TGFα gene and hypomorphic mutations in the EGFR, respectively, have a similar phenotype

(Fowler et al., 1995; Luetteke et al., 1994; Luetteke et al., 1993). Comparably, $EGFR^{-/-}$ mice display strain-dependent defects in epidermal and hair follicle differentiation. They fail to develop a hairy coat, most likely because EGFR signaling is crucial for maintenance of hair follicle integrity (Hansen et al., 1997; Luetteke et al., 1994; Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995). Due to the fact that EGFR-deficient mice have a lifespan of maximally 3 weeks, hair follicle development and cycling could not be carefully studied. Humanized $hEGFR^{KI/KI}$ mice, which express only very low levels of the human EGFR instead of the endogenous mouse receptor in the skin, proved to be extremely beneficial for this purpose and revealed that after hair follicle morphogenesis, EGFR-deficient hair follicles are unable to progress normally through anagen-to-catagen transition and remain in anagen. Hair follicles are progressively degraded, leading to massive infiltration of inflammatory cells, and $hEGFR^{KI/KI}$ mice are completely bald by the age of 6 months (Sibilia et al., 2003). Similar skin and hair follicle malformations are observed in transgenic mice expressing a dominant-negative human EGFR (CD533) in the basal cells of the epidermis (Murillas et al., 1995). Interestingly, these mice also displayed a strong inflammation. This suggests that EGFR signaling may be of physiological relevance in protecting the hair follicle from immunological reactions (Schneider et al., 2008b). Together, these findings reveal that EGFR signaling is crucial for the regulation of hair cycle progression and for preserving hair follicle integrity by controlling the proliferation, differentiation, and survival of epithelial cells.

However, EGFR signaling was also shown to have an inhibitory effect on the control of hair follicle induction. EGF treatment leads to inhibition of hair follicle initiation both *in vivo* and *ex vivo* (Adelson et al., 1997; du Cros, 1993; Kashiwagi et al., 1997). Transgenic mice overexpressing EGF receptor ligands, such as TGFα, amphiregulin, and EGF, in basal epidermal keratinocytes, show fewer hair follicles and retardation of hair follicle development (Mak and Chan, 2003; Vassar and Fuchs, 1991).

Another line of evidence that EGFR is important for the physiological homeostasis of the skin comes from cancer patients treated with EGFR inhibitors who frequently suffer from cutaneous toxicities. EGFR inhibition affects keratinocytes by inducing growth arrest and apoptosis, reducing cell

migration, advancing cell attachment and differentiation, and stimulating inflammation, all of which result in distinctive cutaneous manifestations. Papulopustular rash in the face and upper trunk (45–100%), dry and itchy skin (12–16%), abnormalities in hair growth (21%), particularly the scalp and eyelashes, and inflammation around the nails with tenderness (12–16%) are the most common side effects (Lacouture, 2006). However, in many cases, there is a positive correlation between the occurrence of cutaneous side effects and tumor regression (Perez-Soler and Saltz, 2005).

In summary, EGFR plays an important role in many aspects of cutaneous biology and pathology. EGFR affects proliferation and differentiation of interfollicular and follicular epidermal cells. Moreover, EGFR signaling modulates hair follicle morphogenesis and cycling, and may serve to protect the hair follicle from immunological reactions.

3.4.1.3 EGFR in wound healing

A series of clinical and experimental studies have demonstrated that EGFR signaling is involved in wound healing. One of the first indications that EGFR signaling might have a role in orchestrating the complex processes in wound repair comes from the fact that multiple EGFR ligands have been detected in wound fluid (Grotendorst et al., 1989; Marikovsky et al., 1993; Ono et al., 1995). In addition, upon injury EGFR expression is transiently increased (Stoscheck et al., 1992). Interestingly, it was shown that skin damage results in immediate shedding of EGFR ligands. Furthermore, it was demonstrated that inhibition of metalloproteinases, which cleave the ligand precursors, block keratinocyte migration and impair reepithelialization (Tokumaru et al., 2000). However, even though a large number of EGFR ligands is present at the wound site, not all of them display a strong phenotype in wound repair. Surprisingly, in TGFα knock-out mice wound healing was reported to occur normally, and only on closer examination in an ear wound model where keratinocyte migration and, thus, reepithelialization are crucial, small differences were observed (Kim et al., 2001; Luetteke et al., 1993; Mann et al., 1993). In humans topical application of recombinant EGF resulted in enhanced healing of split-thickness wounds (Brown et al., 1989). A recent study employing transgenic mice that ubiquitously

overexpressed betacellulin, revealed that betacellulin does not affect wound repair, but accelerates wound angiogenesis (Schneider et al., 2008a). Targeted elimination of epiregulin did not affect wound healing. In contrast, epidermal-specific deletion of HB-EGF, which is highly expressed immediately after injury, resulted in a severe delay in wound closure due to impaired keratinocyte migration (Shirakata et al., 2005). Taken together, these findings suggest that HB-EGF is the major and essential EGFR ligand in the process of the reepithelialization process.

Importantly, wound healing studies in EGFR-null and wild-type skin grafts revealed that in incisional wounds EGFR signaling is involved in several early events in the complex process of wound repair including keratinocyte proliferation and migration, reepithelialization, inflammation, and wound angiogenesis (Repertinger et al., 2004). However, these skin grafting experiments were rather artificial. Therefore, it is unclear whether the phenotype seen was due to the grafting or the wound healing response. In addition, several transcription factors known to modulate EGFR expression such as AP-1 or STAT3, were shown to be involved in wound closure (Schafer and Werner, 2007).

The fact that a large number of EGFR ligands is present at the wound site may indicate that there is functional redundancy and compensation in the skin to assure proper wound healing. Thus, to unravel how EGFR signaling accounts for the wound healing process, detailed wound repair studies will have to be performed in conditional EGFR mice, which would allow epidermis-specific deletion of the receptor.

3.4.1.4 Role of EGFR during skin tumorigenesis

Skin cancer is the third most common human malignancy, and its occurrence has been increasing rapidly over the past decades, with basal cell carcinoma (BCC), squamous cell carcinoma (SCC), and melanoma being the most common forms. An estimated number of 2–3 million non-melanoma skin cancer patients and 132,000 patients of melanoma are counted every year (World Health Organization). While in human SCCs amplification of the EGFR is very common (Maubec et al., 2005; Nicholson et al., 2001), focal amplification and/or

mutation of EGFR have not been reported in melanomas. However, late-stage melanomas frequently display EGFR overexpression in association with extra copies of chromosome 7, where the human EGFR gene is localized (Chin et al., 2006). A huge number of studies in mouse models reveal that alterations in the EGFR pathways result in epithelial neoplasm including those induced by two-stage carcinogenesis in mouse skin where activation of Ha-Ras is a critical event in papilloma formation (Frame et al., 1998). Topical administration of distinct tumor promoters on mouse skin causes elevated levels of EGFR and its ligands TGFα, AR, and HB-EGF in developing primary papillomas and SCC (Kiguchi et al., 1998). Interestingly, TGFα overexpression in basal or suprabasal epidermal cells results in thickening of the epidermis and papilloma development preferentially at sites exposed to mechanical irritation, and TGFα expression can bypass the need for chemically induced Ha-Ras mutations (Dominey et al., 1993; Vassar et al., 1992; Wang et al., 1994). Furthermore, overexpression of ErbB2 in the basal layer of the epidermis leads to the formation of spontaneous papillomas with the ability to convert to SCC within the first 6 weeks of age (Kiguchi et al., 2000). In addition, a reduction in the size of papillomas derived from EGFR null keratinocytes expressing v-ras^{Ha} in grafting experiments has been reported (Dlugosz et al., 1997). Constitutive expression of an activated form of Ha-Ras in suprabasal epidermal cells induces the development of benign papillomas in transgenic mice at sites of promotional stimuli (Bailleul et al., 1990). Moreover, transgenic mice expressing an activated Ras in the outer root sheaths of hair follicles develop spontaneous papilloma-like structures, which frequently convert to malignant SCC (Brown et al., 1998). On the contrary tamoxifen-inducible activation of a K14-Ras transgene in mice leads to massive cutaneous hyperplasia and suppressed differentiation that is reversible upon withdrawal of tamoxifen treatment (Tarutani et al., 2003).

The analysis of transgenic mice expressing a constitutively active form of the Ras activator Son of Sevenless (SOS) in the basal cells of the epidermis (K5-SOS mice) unraveled the role of EGFR especially in the early steps of skin tumor development. These mice develop skin papillomas at 100% penetrance in a wild-type EGFR background. However, tumor formation is severely impaired when these mice are bred into an EGFR hypomorphic background.

K5-SOS transgenic keratinocytes and papillomas from $EGFR^{wa2/wa2}$ mice are more differentiated and display reduced Akt phosphorylation and increased apoptosis, suggesting that the EGFR functions as a survival factor during oncogenic transformation by components of the Ras signaling pathway (Sibilia et al., 2000). Interestingly, a recent study revealed that survival of cancer cells may be maintained by EGFR independent of its kinase activity (Weihua et al., 2008). Mice lacking c-Jun in the epidermis display reduced expression of EGFR in basal keratinocytes, and K5-SOS-dependent skin tumor formation is strongly reduced. This study clearly demonstrated that in the skin Jun regulates EGFR expression at the transcriptional level (Zenz et al., 2003). Interestingly, patients with dominant Hereditary Gingival Fibromatosis type 1 carry a frameshift mutation in the SOS1 gene, leading to a truncated SOS protein similar to the one expressed in K5-SOS mice (Hart et al., 2002). This highlights the relevance of K5-SOS transgenic mice as a model for human cancer and for anti-tumor therapies.

Importantly, EGFR-null fibroblasts are also resistant to transformation by SOS and RasV12 (Sibilia et al., 2000). Furthermore, EGFR-deficient keratinocytes expressing $v\text{-}ras^{Ha}$ develop smaller papillomas when grafted into immunodeficient mice (Dlugosz et al., 1997). Similar results were obtained with Ha-Ras transgenic animals expressing dominant-negative EGFR (K5.dnEGFR) in basal keratinocytes. Whereas tumor onset was similar in mice expressing dnEGFR and controls, tumor volumes were much smaller and had a pale appearance in K5.dnEGFR transgenic mice (Casanova et al., 2002). Interestingly, these papillomas show reduced Akt activity and increased numbers of apoptotic cells. Furthermore, abrogation of EGFR function was shown to result in a dramatic decrease in vascular endothelial growth factor (VEGF) expression and an altered angiogenic response unable to properly nourish and oxygenate tumor cells, which may thus account for the decline in cell survival. These findings provide compelling functional evidence that, in addition to the Ras/MAPK pathway, an EGFR-dependent pathway acting via Akt is essential for the transformation of mouse epidermal cells (Segrelles et al., 2002). Whether a similar mechanism is acting in human SCC remains to be investigated.

Recent studies provide evidence that mitogen-inducible gene 6 (Mig6, also known as RALT) is a specific negative regulator of EGFR signaling in skin morphogenesis and a novel tumor suppressor of EGFR-dependent carcinogenesis. Its expression is downregulated in various human cancers (Ballaro et al., 2005; Ferby et al., 2006). Mice deficient for the gene encoding Mig6 (*errfi1*; ErbB receptor feedback inhibitor 1) display hyperactivation of endogenous EGFR resulting in hyperproliferation and impaired differentiation of epidermal cells. Furthermore, mice spontaneously develop tumors in various organs and are highly susceptible to chemically induced formation of skin tumors. Interestingly, breeding of Mig6 knock-out mice into an EGFR hypomorphic background or treatment with Gefitinib completely rescues the skin defects and tumor development is inhibited. Therefore, Mig6 acts as a tumor suppressor in EGFR-dependent carcinogenesis (Ballaro et al., 2005; Ferby et al., 2006). The transcription factor AP-2α is also often reduced in tumor cells and was recently shown to act as a tumor suppressor as well (Friedrichs et al., 2005). Deletion of AP-2α in mice recapitulates the phenotype of Mig6 knock-out mice. AP-2α deficient epidermis exhibits elevated levels of EGFR expression and Akt activity in the differentiating layers, resulting in hyperproliferation and papilloma-like invaginations. These data indicate that AP-2α controls epidermal cell proliferation and differentiation, and functions by repressing EGFR expression as keratinocytes exit the basal layer and commit to terminally differentiate (Wang et al., 2006b).

Altogether these findings underscore the pivotal role of EGFR and its family members in epithelial tumor development and highlight the importance of EGFR and the upstream and downstream molecules of its complex signaling pathway as a target for therapeutic intervention in epithelial tumors.

3.4.1.5 Targeted EGFR inhibitors

The ErbB receptors were shown to be aberrantly activated in many human tumors, and therefore they are excellent targets for anti-cancer therapies. Currently, two types of ErbB inhibitors are in clinical or pre-clinical use: antibodies like Cetuximab (Erbitux) and Trastuzumab (Herceptin) directed

against EGFR and ErbB2, respectively, and small molecule tyrosine kinase inhibitors such as Erlotinib (Tarceva), or Lapatinib (Gschwind et al., 2004; Hynes and Lane, 2005). Treatment of tumors with these drugs had an impact on many of the intracellular signal transduction pathways involved in tumor formation and progression. In pre-clinical models as well as in tumors from cancer patients treatment with both ErbB-targeted tyrosine kinase inhibitors and antibodies affected PI3K–Akt, MAPK, Src and STAT signaling, and consequently tumor progression (Gschwind et al., 2004; Hynes and Lane, 2005; Wieduwilt and Moasser, 2008). However, since signaling mediated by the EGFR and its family members is also required for physiological processes, anti-ErbB therapy is frequently associated with side effects. The most obvious ones are cutaneous toxicities such as papulopustular rash, dry and itchy skin and hair growth defects found in 45-100% of patients. The subsequent physical and psychological discomfort frequently leads to interruption of anti-cancer therapy (Lacouture, 2006).

During the process of cancer development multiple genetic alterations arise in tumor cells which contribute to the metastatic phenotypes of fully malignant tumors. The ErbB family was shown to be involved in many of the processes leading from benign to malignant cancers. However, treatment with inhibitors targeting the ErbB receptors was not always successful. Tumor cells were often found to acquire resistances to EGFR-targeted tyrosine kinase inhibitors, for instance by gain-of-function mutations in the kinase domain or loss or mutation of the negative regulator of PI3 kinase PTEN (Hynes and Lane, 2005). Upregulation of c-Met and IGF1R were also reported in tumors resistant to anti-EGFR therapies (Bean et al., 2007; Chakravarti et al., 2002; Engelman et al., 2007). Therefore, a combination of inhibitors targeting different signaling pathways is probably the key to success in cancer therapy.

3.4.2 The vascular endothelial growth factor (VEGF) and its receptors in skin cancer

Tumors and metastases usually arise as small avascular masses which subsequently induce neovascularization in order to acquire nutrients for continued growth and metastatic spread. Therefore, tumor cells secrete angiogenic factors, which induce the angiogenic switch, thereby allowing tumors to progress (Bergers and Benjamin, 2003; Hirakawa et al., 2005). One of the key regulators of tumor angiogenesis is the vascular endothelial growth factor A (VEGF). Many years of substantial research have implicated a major role of the VEGF family in orchestrating angiogenesis (Ferrara, 2002). VEGF (also referred to as VEGF-A) was originally termed vascular permeability factor (VPF) because of its ability to induce vascular leakage (Senger et al., 1983). It belongs to a gene family that includes placental growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D and VEGF-E. Alternative exon splicing of VEGF results in different isoforms: 3 transcripts in mice ($VEGF_{120}$, $VEGF_{164}$, $VEGF_{188}$; Fig. 7) and 9 isoforms in humans (Ferrara et al., 2003; Takahashi and Shibuya, 2005).

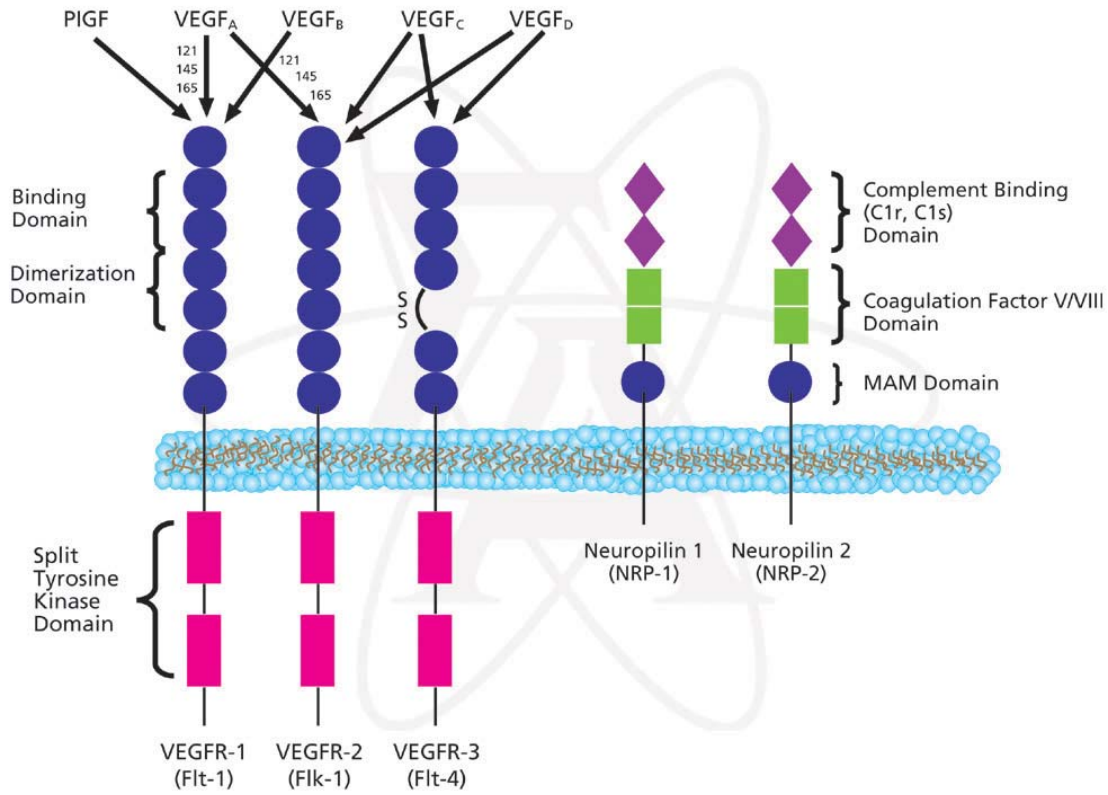

Figure 7. Schematic representation of the VEGF family of ligands and receptors (adapted from Sigma Aldrich).

While in mice $VEGF_{120}$ diffuses freely in the surrounding extracellular matrix, the other isoforms show increased binding to the heparin-rich extracellular matrix (Carmeliet and Collen, 1999). VEGF binds to two related receptor tyrosine kinases, VEGFR1/Flt1 and VEGFR2/KDR/Flk1, both of which are primarily expressed on vascular endothelial cells (ECs; Fig. 7,8) and their expression is upregulated during angiogenic processes. VEGFR1/Flt1 and VEGFR2/Flk1/KDR comprise seven immunoglobulin-like domains in the extracellular domain, a single transmembrane region and a consensus tyrosine kinase sequence that is interrupted by a kinase insert domain (Shibuya et al., 1990; Terman et al., 1991). Both receptors can transduce signals of other growth factors (Fig. 7) but only the VEGF isoforms are capable of binding to both VEGFR1 and VEGFR2 (Neufeld et al., 1999). Upon ligand binding the receptors undergo dimerization, which results in ligand-dependent autophosphorylation of different tyrosine residues located in the C-terminal region of the receptor. Moreover, VEGF interacts with a family of coreceptors, the neuropilins (Nrp), which were originally identified to be implicated in axonal guidance and have recently been shown to enhance VEGFR signaling (Ferrara et al., 2003) (Fig.7,8). Since the cytoplasmic domain of Nrp1 is short, it was originally suggested that Nrp1 is unable to transduce biological signals (He and Tessier-Lavigne, 1997). Now it is apparent that Nrp1-mediated angiogenesis occurs via G-protein signaling molecules (Murga et al., 2005; Wang et al., 2006a).

VEGF has been identified as a survival factor for endothelial cells and has been shown to be indispensable for embryonic angiogenesis and vasculogenesis (Gerber et al., 1999; Lee et al., 2007a). Disruption of both VEGF alleles in mice results in almost complete absence of a vasculature, and already the inactivation of one single VEGF allele is embryonic lethal due to abnormal vasculature (Carmeliet et al., 1996; Ferrara et al., 1996). Likewise, $VEGFR1^{-/-}$ and $VEGFR2^{-/-}$ mice die *in utero* due to abnormal vascular channels or early defects in the development of hematopoietic and endothelial cells, respectively (Fong et al., 1995; Shalaby et al., 1995). Conditional deletion of VEGF within the first postnatal weeks in various organs such as heart, kidney, liver, spleen, bone marrow, and brain results in severe vascular aberrances and lethality. On the contrary, VEGF inactivation in adult animals is much less traumatic

indicating that VEGF does not have a continuous maintenance function for most of the adult vasculature (Gerber et al., 1999). However, genetic deletion of VEGF in the endothelial lineage leads to progressive endothelial degeneration and sudden death in mutant mice by 25 weeks of age suggesting that autocrine VEGF signaling is essential for vascular homeostasis (Lee et al., 2007a).

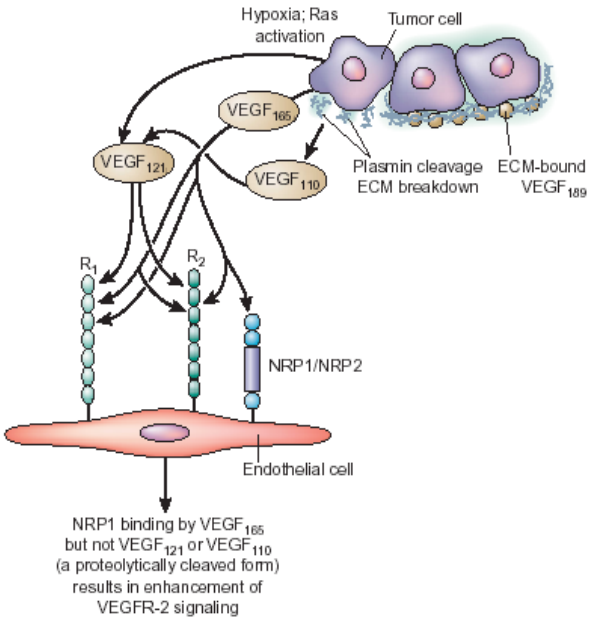

Figure 8. The VEGF isoforms and their interaction with VEGF receptors. In response to a variety of stimuli, the diffusible isoforms, VEGF120 and VEGF164, are released by normal and transformed cells and may bind to VEGFR1 and VEGFR2. VEGF164 also interacts with NRP1 and NRP2. This binding enhances the VEGFR signaling in endothelial cells. Following plasmin generation and ECM breakdown, VEGF188 is cleaved at the C-terminus, and the resulting 110aa N-terminal fragment is diffusible and bioactive (adapted from Ferrara, 2003).

Interestingly, overexpressing of $VEGF_{120}$ in the suprabasal compartment of the epidermis via the keratin 6 promoter in mice resulted in skin swelling, erythema and edema subsequently leading to the disruption of skin architecture and early postnatal lethality (Larcher et al., 1998). Furthermore, VEGF overexpression in the skin of transgenic mice expressing Ha-ras resulted in accelerated tumor formation (Larcher et al., 1998). It is therefore evident that VEGF is a potent vascular regulator and that its dosage must be exquisitely regulated in a spatial, temporal and quantitative manner to avoid vascular disorders.

VEGF overexpression is found in many human and murine tumors such as those of epithelial origin (Dvorak, 2002; Larcher et al., 1996). In tumors VEGF upregulation goes along with hypoxia and/or overexpression of a variety of

oncogenes including mutant ras, bcr-abl, ErbB2/Her2, and activated EGFR. The classical role for VEGF expressed by tumor cells is that it acts on neighboring endothelial cells thereby promoting tumor vascularization (Bergers and Benjamin, 2003; Ferrara et al., 2003; Ferrara and Kerbel, 2005). For instance, during two-stage skin chemical carcinogenesis in mice it could be demonstrated that EGFR signaling is responsible for Ha-ras-dependent VEGF upregulation and induction of the angiogenic switch necessary for tumor growth (Casanova et al., 2002). In this model, expression of dominant negative EGFR in tumors could inhibit blood vessel remodeling suggesting that a profound reduction of VEGF expression is the critical event responsible for angiogenesis and tumor growth suppression. Accordingly, deletion of VEGF in basal layers of the epidermis impaired carcinogen-induced papilloma formation (Rossiter et al., 2004). Recent studies demonstrated expression of VEGFR1 and VEGFR2 as well as Nrp1 on tumor cells (Chung et al., 2006; Fakhari et al., 2002; Lee et al., 2007b; Parikh et al., 2004). Although *in vitro* it could be shown that VEGFR signaling can mediate intracrine survival of tumor cell lines (Lee et al., 2007b), the expression and function of VEGFRs in tumors *in vivo* remains controversial.

4 GOALS OF THE THESIS

The objectives of the research done during my PhD studies were to investigate the function of the EGFR in the skin, and to analyze whether EGFR mutant mice die as a consequence of epithelial defects such as impaired skin barrier function. Moreover, by employing conditional EGFR (floxed) mice, which allow epidermis-specific deletion of the receptor, I aimed to investigate how EGFR signaling affects hair follicle morphogenesis and hair follicle cycling, since a detailed analysis could not yet be performed due to the short lifetime of EGFR-null mice. More importantly, these mice also allowed to study the impact of EGFR on wound healing.

Another goal of the thesis was to examine the role of EGFR signaling during initiation and progression of SOS-dependent skin tumor development. Interestingly, epidermal cells isolated from K5-SOS transgenic mice express high levels of the vascular endothelial growth factor (VEGF) and β1 integrin, which are key players of tumor angiogenesis and tumor cell migration, respectively. There are several in vitro evidences for a crosstalk between EGFR and β1 integrin, but the *in vivo* relevance of this interaction has never been analyzed. Therefore I examined the impact of β1 integrin deletion on SOS-dependent skin carcinogenesis and studied the cellular and molecular mechanisms by which signaling events downstream of EGFR and/or β1 integrin control the underlying biological processes. Furthermore, I investigated how conditional deletion of VEGF in epidermal cells, which are its major source in the skin, affects skin tumorigenesis, and whether EGFR and VEGF cooperate in tumor cells to promote epithelial cancers.

5 RESULTS AND DISCUSSION

5.1 EGFR function in epithelial development and wound healing

5.1.1 EGFR mutant mouse strains

To address the role of EGFR in skin physiology and pathology I used different EGFR mutant mouse strains, which will be briefly described here.

$EGFR^{-/-}$ mice display strain-dependent phenotypes with defects in neural and epithelial tissues and die at different stages of embryonic and early postnatal development depending on their genetic background (Miettinen et al., 1995; Sibilia et al., 2007; Sibilia et al., 1998; Sibilia and Wagner, 1995; Threadgill et al., 1995).

In $hEGFR^{KI/KI}$ mice the endogenous mouse EGFR was replaced by the human receptor by a knock-in approach (Sibilia et al., 2003). Due to very low expression levels of EGFR in the skin, $hEGFR^{KI/KI}$ mice display severe epithelial defects which resemble those of EGFR null mice. However, in contrast to the very short lifespan seen in $EGFR^{-/-}$ mice, $hEGFR^{KI/KI}$ mice can live up to six months and die because of heart defects.

Moreover, I made use of conditional EGFR mice, which were generated in our laboratory (Natarajan et al., 2007). These carried either floxed or flirt alleles. The flirt allele is actually a floxed EGFR allele that still harbors a Neo-cassette, which can be deleted by Flp-mediated recombination. Since both alleles behave the same and result in a delta (Δ) allele lacking the promoter and exon 1 of EGFR upon Cre-mediated recombination, I do not discriminate between the two alleles and both are indicated as $EGFR^{f/f}$ hereafter. $EGFR^{f/f}$ were crossed to K5-Cre (Tarutani et al., 1997) and K5-$CreER^{T}$ (Indra et al., 1999) transgenic lines to generate mice in which EGFR is constitutively deleted in the basal layers of the epidermis starting from embryonic day 14.5 ($EGFR^{\Delta ep}$), or mice in which EGFR deletion could be induced by administration of tamoxifen ($EGFR^{\Delta epER}$), respectively.

Furthermore, $EGFR^{\Delta ep}$ and $hEGFR^{KI/KI}$ mice were crossed to hairless (hr/hr) and $Rag2^{-/-}$ mice. Hairless mice carry a mutation in the hairless (hr) gene and progressively loose hair becoming completely bald 3-4 weeks after birth (Brancaz et al., 2004). Very mild or no immunological defects have been

described for these mice. In contrast, Rag2$^{-/-}$ mice develop normal hair but are immunodeficient due to a lack of mature T- and B-cells (Shinkai et al., 1992).

5.1.2 Mice lacking EGFR expression in the basal layer of the epidermis have a similar phenotype as EGFR-null mice

EGFR knock-out mice exhibit severe epithelial defects and develop a progressive neurodegeneration, and die within the first postnatal days or already during embryogenesis depending on the genetic background. Recent results obtained in our laboratory show that transgenic expression of a constitutively active Ras (RasV12) in postmitotic neurons rescues the neurodegeneration of EGFR$^{-/-}$ mice but does not prolong their lifespan (Wagner et al., 2006). On the contrary, transgenic expression of a constitutively active form of the Ras nucleotide exchange factor Son of Sevenless from the keratin 5 promoter (K5-SOS) which is active in all stratified epithelia such as skin and gastro-intestinal (GI)-tract rescues the lethality of EGFR$^{-/-}$ mice but the mice still develop the brain degeneration. These results suggest that the lethality of EGFR mutants is most likely due to epithelial rather than neural defects.

To study the function of the EGFR in epithelia, mice lacking the EGFR in the basal layers of all stratified epithelia such as skin and gastrointestinal (GI)-tract (EGFR$^{\Delta ep}$) were generated in our laboratory by crossing mice carrying conditional EGFR alleles (EGFR$^{flox/flox}$, EGFR$^{f/f}$) (Natarajan et al., 2007) with a K5-Cre transgenic line (Tarutani et al., 1997). Interestingly, these mice developed a phenotype similar to EGFR$^{-/-}$ mice (Fig. 9A-G). EGFR$^{\Delta ep}$ mutants were born at almost Mendelian ratio (21,5%), their eyes were open at birth (Fig. 9A), they were growth retarded (Fig. 9C,G) and they failed to develop a hairy coat (Fig. 9B,C). Morover, 90% of EGFR$^{\Delta ep}$ mutant mice did not survive longer than one month after birth (Fig. 9F).

To rule out that EGFR is also deleted in other tissues in EGFR$^{\Delta ep}$ mice due to aberrant K5-Cre expression, EGFR deletion and expression were analyzed in different organs of EGFR$^{\Delta ep}$ mice and controls at the genomic and protein level, respectively. Southern blot analysis revealed that EGFR was deleted to more than 90% in total skin and tail biopsies, whereas no deletion could be detected in other tissues (Fig. 9D). While EGFR$^{\Delta ep}$ mice showed high EGFR protein

expression levels in various tissues such as brain, lung and liver, the expression was significantly reduced in the skin when compared to the expression levels in littermate controls (Fig. 9E). These results confirm that the EGFR was efficiently deleted in the epidermis. The remaining EGFR expression found in the skin most likely derives from dermal fibroblasts or immune cells present in the skin.

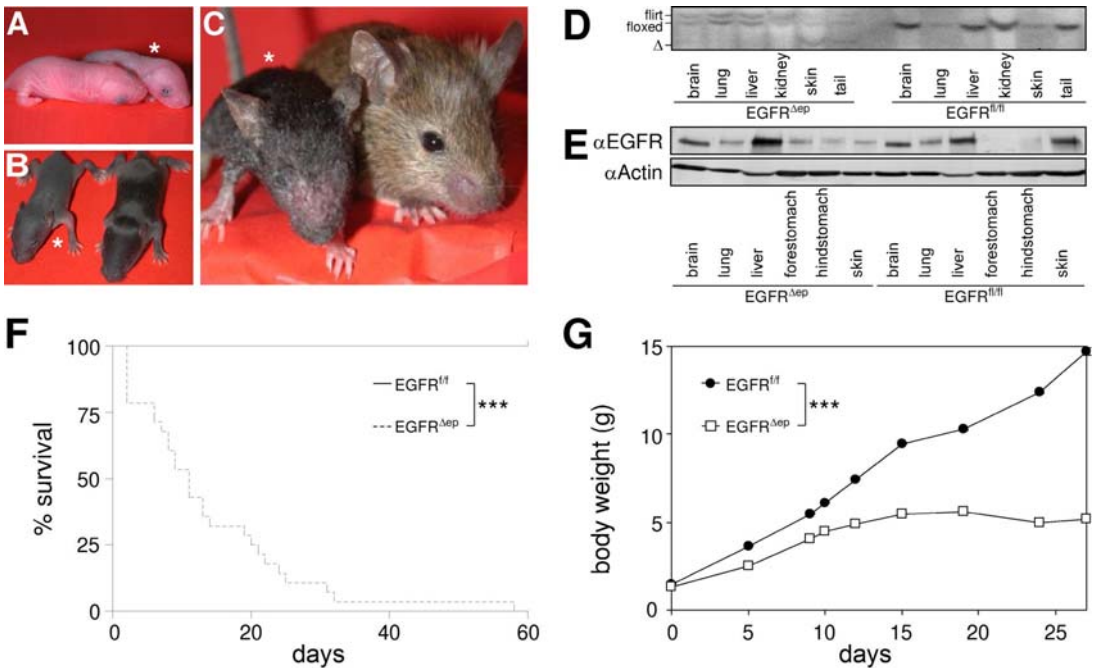

Figure 9. Epidermis-specific deletion of EGFR leads to early postnatal lethality. (A,B,C) Phenotype of EGFR$^{\Delta ep}$ mutants (indicated by an asterisk) and control littermates at postnatal day 2 (P2; A), P10 (B) and P20 (C). **(D)** Southern blot analysis showing tissue-specific deletion of EGFR in EGFR$^{\Delta ep}$ mice. Upon Cre-mediated recombination the floxed or flirt - a floxed allele containing a Neo-cassette - alleles result in a Δ allele. **(E)** Western blot analysis showing EGFR expression in various tissues of EGFR$^{\Delta ep}$ and control mice. **(F)** Kaplan-Meier curve showing reduced survival in EGFR mutants (n=28). **(G)** Body weight of EGFR$^{\Delta ep}$ mice is significantly reduced (n=8). Data represent mean ± SEM. *** p≤0.0005.

At birth, EGFR$^{\Delta ep}$ mice had a similar body weight as their littermate controls. However, the weight gain was severely impaired later on and resulted in dramatic weight loss within the third and forth week (Fig. 9G). Since the K5 promoter is also active in epithelial tissues of the intestine, the strong weight loss might be due to EGFR deletion in the GI-tract. These results suggest that EGFR$^{\Delta ep}$ mice die because of a functionally impaired intestine.

Next I investigated if EGFR mutant mice suffer from a defect in the barrier function of the skin which would eventually lead to enormous water loss or sepsis by invading microbes. Usually, the skin barrier forms in a patterned manner starting around day 16 during embryogenesis (E16). Specific skin sites on both sides of the spinal column acquire impermeable characteristics, and

then a moving front of impermeability spreads across the animal's body (Hardman et al., 1998). A qualitative *in situ* assay for skin permeability revealed a delay in the skin barrier formation both in EGFR$^{-/-}$ (Fig. 10A-F) and EGFR$^{\Delta ep}$ mice (Fig. 10G-K).

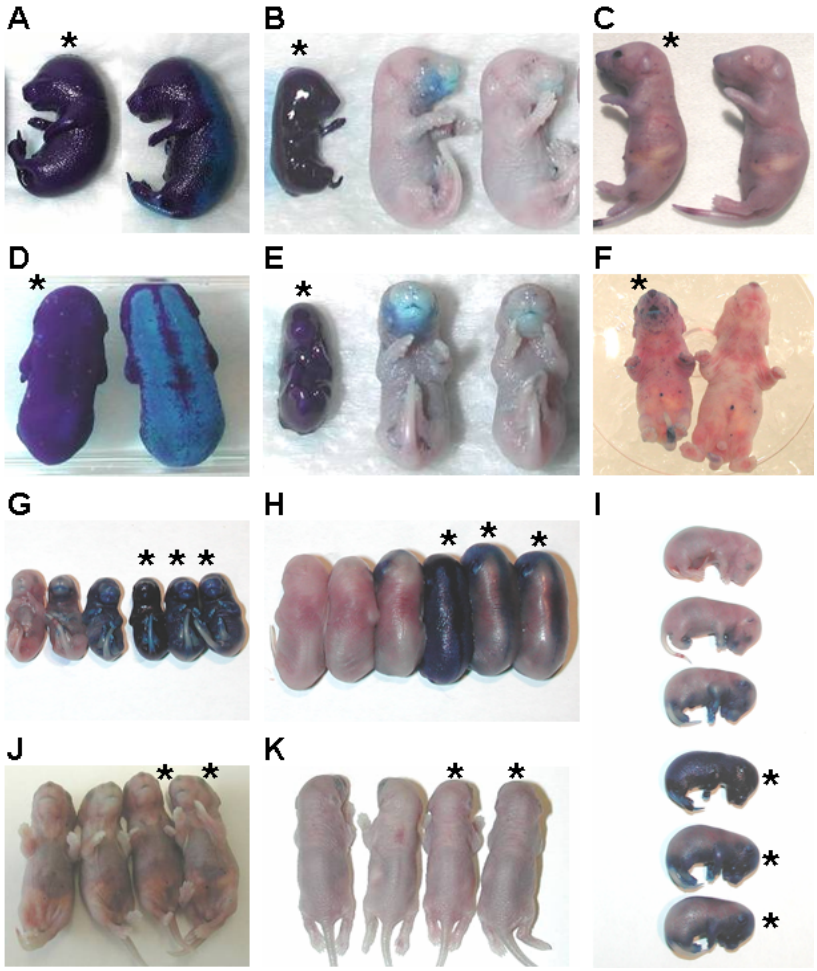

Figure 10. EGFR mutant mice display a delay in skin barrier formation. Toluidine blue staining of EGFR$^{-/-}$ mutants (indicated by an asterisk) and control littermates at embryonic day E16.5 **(A,D)**, E17.5 **(B,E)** and postnatal day P1 **(C,F)**, and of EGFR$^{\Delta ep}$ mutants at E17 **(G-I)** and P1 **(J,K)**. EGFR$^{\Delta ep}$ mice (indicated by an asterisk) do not show a skin barrier defect after birth (P1) **(J,K)**.

While in EGFR wild-type mice the skin barrier started to form already at E16.5 and was almost completed by E17.5, the skin of EGFR$^{-/-}$ fetuses was still completely permeable at these stages (Fig. 10A,B,D,E), and even after birth permeable areas persisted in the face (Fig. 10F). In EGFR$^{\Delta ep}$ mutants, a delay in skin barrier formation was also observed, but the phenotype was milder than in EGFR-null mice. Toluidine blue staining at E17 showed either no or only mild dorsal formation of the skin barrier in EGFR$^{\Delta ep}$ mice, while the skin of littermate controls was either completely impermeable or had only partial ventral staining

(Fig. 10G-I). However, EGFR$^{\Delta ep}$ mutants did not show any skin barrier defects at postnatal day 1 (Fig. 10J,K).

These results suggest that the lack of EGFR expression in the epidermis is lethal and EGFR mutant mice display derangements of the skin and hair follicles. Moreover, complete or epidermis-specific deletion of the receptor delays the formation of a skin barrier.

5.1.3 Epidermis-specific deletion of EGFR in adults results in a mild phenotype

To investigate whether the EGFR is essential in epithelia of adult mice, I generated mice in which the EGFR could be conditionally deleted in a temporal manner by employing the tamoxifen-inducible K5-Cre-ER^{T2} transgenic line (Indra et al., 1999).

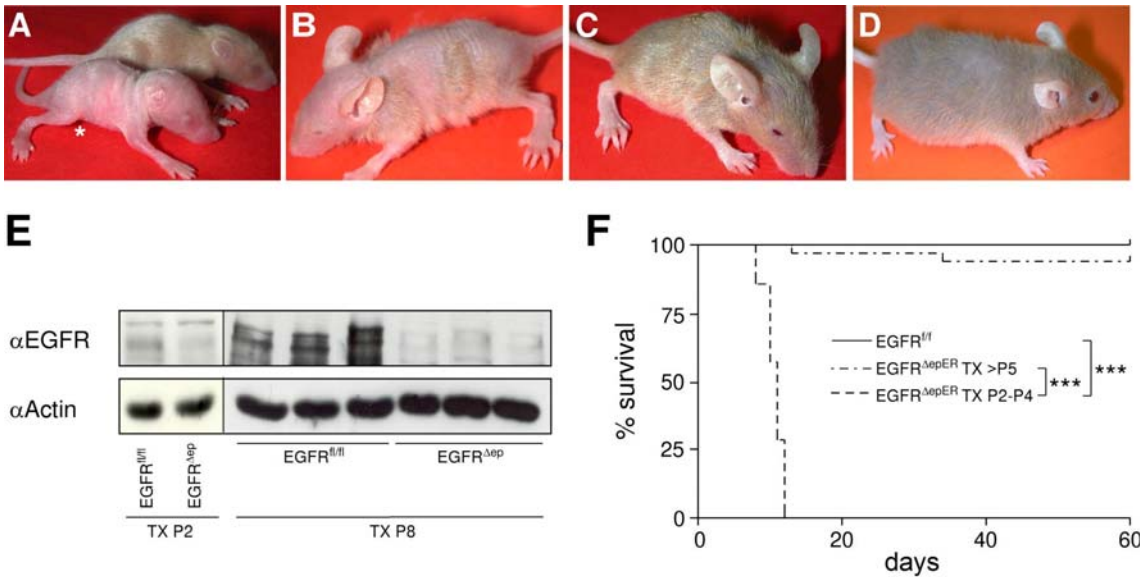

Figure 11. EGFR deletion in adult skin is not lethal. (A-D) Phenotype of EGFR$^{f/f}$ K5-Cre-ER^{T2} (=EGFR$^{\Delta epER}$) mice upon intraperitoneal tamoxifen administration starting at P2 (A) or P5 (B-D). Pictures were taken at P6 (A), P25 (C), P45 (D) and P130 (E); (C-D) represent the same mouse. **(E)** Western blot analysis of protein lysates from epidermis isolated from EGFR$^{\Delta epER}$ and control mice which were treated with tamoxifen from P2 to P10 (lanes 1 and 2), or for several weeks starting from P8 (lanes 3-8). **(F)** Kaplan-Meier plot showing early lethality of EGFR$^{\Delta epER}$ mice upon tamoxifen treatment within the first 4 days after birth. *** p≤0.0005.

Interestingly, intraperitoneal tamoxifen injection before postnatal day 5 resulted not only in a failure to develop a hairy coat but also early lethality (Fig. 11A,F). However, conditional deletion of the EGFR after postnatal day 5 revealed only

macroscopic changes of the skin, such as hair loss or a curly hair phenotype and mice were viable (Fig. 11B-D,F), suggesting that a functional EGFR is essential in epithelia in the first postnatal days. Animals which had turned bald developed a rudimentary hairy coat with reduced hair follicle numbers and wavy hair fibres later on. Analysis of primary keratinocytes isolated from $EGFR^{f/f}$ K5-$Cre\text{-}ER^{T2}$ (=$EGFR^{\Delta epER}$) mice treated with tamoxifen at various time points revealed that even though the EGFR is efficiently deleted in the epidermis at the genomic level, the EGFR protein is very stable on the cell surface. However, approximately 10 days after treatment with tamoxifen EGFR protein expression could no longer be detected (Fig. 11E).

These results suggest that the EGFR is required in the skin in the first postnatal days to guarantee later survival. In contrast, EGFR deletion in adult mice leads to hair growth defects but is not lethal.

5.1.4 Lack of EGFR delays hair follicle morphogenesis and cycling

EGFR mutant mice as well as mice lacking the expression of EGFR ligands or overexpressing them have suggested that EGFR signaling affects hair follicle morphogenesis and controls the entry into anagen-to-catagen transition. However, the early lethality of EGFR deficient mice made the investigation of the impact of EGFR signaling on HF cycling impossible.

Histological analysis of paraffin-embedded skin samples from $EGFR^{\Delta ep}$ (and $EGFR^{-/-}$) mice isolated at critical stages of HF morphogenesis and HF cycling showed a delay in HF cycling (Figure 12A-L). At P1 the morphology of $EGFR^{\Delta ep}$ skin was overtly normal but the number of epidermal layers was reduced when compared to littermate controls (Fig. 12A,G). At P8, when HF morphogenesis is completed, the normal skin morphology was altered and HF were diffusely distributed and disoriented within the dermal compartment (Fig. 12B,H). EGFR mutant hair shafts displayed increased pigmentation and the regular melanin pattern was lost (Fig. 12B,H,M-O). Furthermore, the shape of EGFR mutant hair shafts was irregular. That the diameter of hair shafts is not constant within a HF most probably results from the fact that the inner and outer root sheath (IRS and ORS) of the mutant HF are separating, thereby altering the stability that is necessary for proper hair shaft development (Fig. 12H,I; indicated by a blue

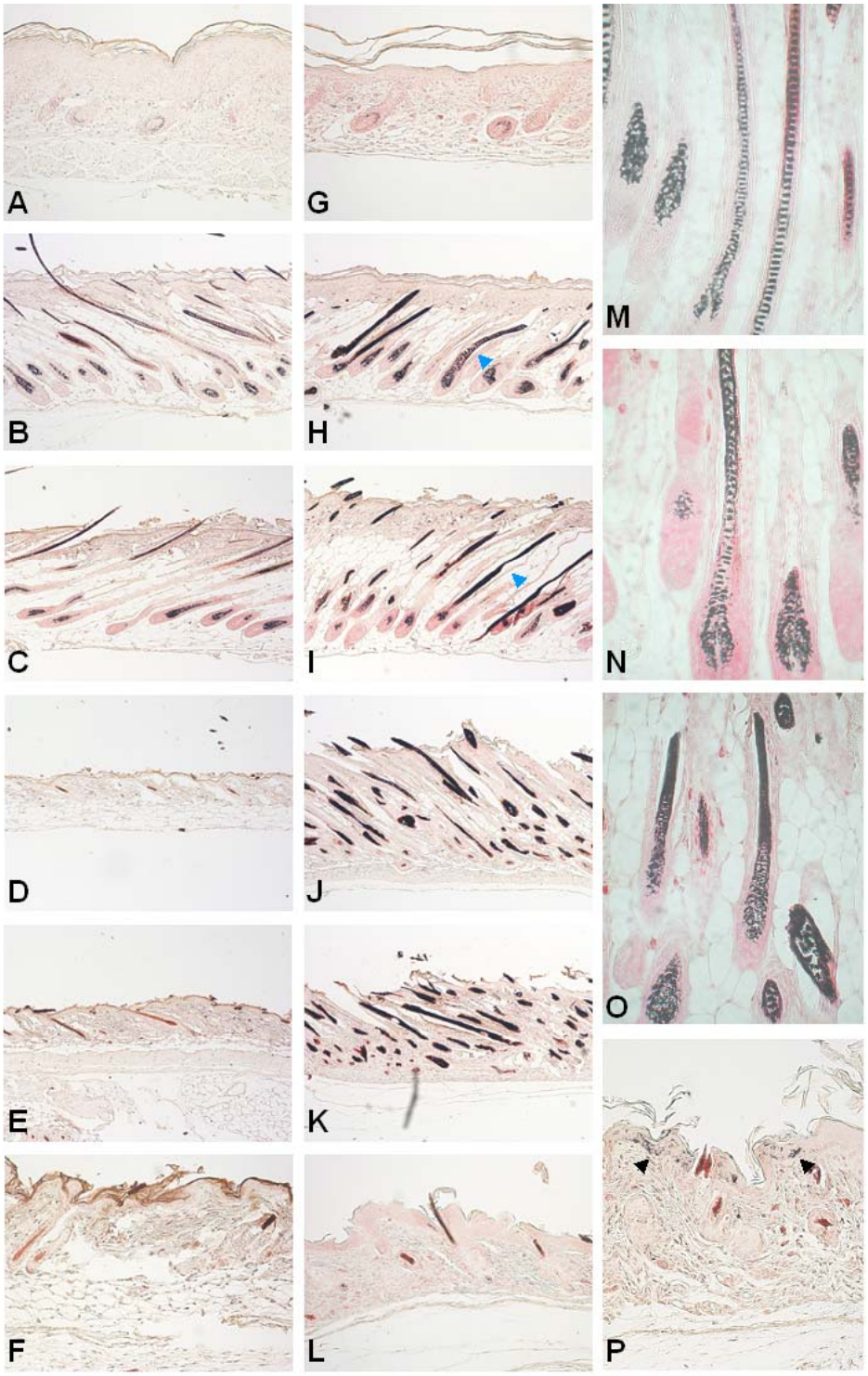

Figure 12. HF morphogenesis and entry into HFcycling is delayed in EGFR$^{\Delta ep}$ mice. **(A-P)** Fontana-Masson staining of paraffin embedded skin samples from EGFR$^{\Delta ep}$ mice (G-L) and littermate controls (A-F) at postnatal day P1 (A,G), P8 (B,H), P10 (C,I), P17 (D,J), P19 (E,K), and P21 (F,L). EGFR$^{\Delta ep}$ mice show a delay in hair follicle morphogenesis. The reason for the irregular shape of EGFR$^{\Delta ep}$ hair follicles (HF) is most probably the separation of the inner and outer root sheaths indicated by the blue arrowheads (H,I). Mutant HF are more and irregularly pigmented (N,O) than control HF (M). At P25 normal skin morphology is distorted in EGFR$^{\Delta ep}$ mice and the skin shows high infiltrate of inflammatory cells. Arrowheads indicate melanin incontinence (P).

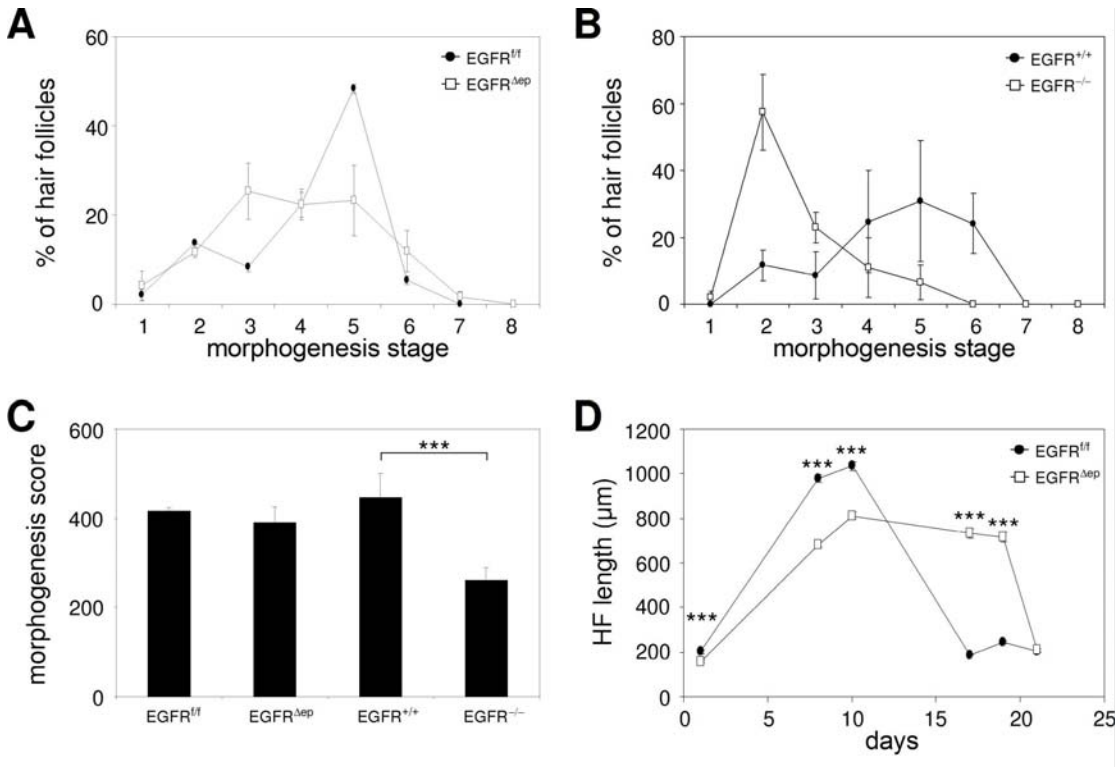

Figure 13. Delayed entry into HF cycling in EGFR mutant mice. (A-D) Quantitative analysis of HF morphogenesis stages from skin samples from $EGFR^{\Delta ep}$ (A) and $EGFR^{-/-}$ mutant mice (B) and their littermate controls isolated at postnatal day P1. The graph shows the percentage of HF in different morphogenesis stages. The average morphogenesis score (C) is calculated by multiplying the percentage of HF in a specific morphogenesis stage with the number of the stage (1-8). (D) Average length of HF from $EGFR^{\Delta ep}$ mutant and $EGFR^{f/f}$ mice at the indicated time points. Data represent mean ± SEM. *** p≤0.0005.

arrowhead). At later time points, the morphology of EGFR mutant skin was increasingly altered (Fig. 12I,J,K,L,P). Whereas wild-type HF had already entered telogen, EGFR mutant HF were still in early catagen at P17 (Fig. 12C, I) and entered telogen only 4 days later (Fig. 12L). Interestingly, EGFR mutant skin displayed melanin incontinence, which is never seen in healthy mouse skin (Fig. 12P; indicated by a black arrowhead).

Next, I performed qualitative and quantitative analyses of HF at different stages of morphogenesis on Fontana-Masson stained paraffin sections of controls and $EGFR^{\Delta ep}$ and $EGFR^{-/-}$ skin samples. Interestingly, $EGFR^{\Delta ep}$ skin contained more HF in early morphogenesis stages than controls at P1. Whereas 63% of the HF in $EGFR^{\Delta ep}$ skin were in stages 2-4 and only 37% had reached the late stages 5 and 6, 53% of wild-type HF had already entered stages 5 or 6 (Fig. 13A). This phenomenon was even more pronounced in $EGFR^{-/-}$ mice where 93% HF were still in early to mid morphogenesis stages (Fig. 13B,C). Staging of HF was impossible at later time points due to the irregular distribution and orientation of

HF. Therefore the average HF length was measured. Wild-type HF had fully developed until postnatal day 10 reaching their maximal length of approximately 1000µm and underwent apoptosis-driven organ involution to enter the quiescent telogen phase around P17. In contrast, HF of $EGFR^{\Delta ep}$ mice - even though their morphogenesis was completed at P8 - remained stuck in their development after P8 and only slowly entered catagen between P17 and P19 (Fig. 13D). In addition, they never reached the size of wild-type HF.

To study the effect of EGFR deletion after HF morphogenesis, I induced EGFR deletion by intraperitoneal tamoxifen injection starting from postnatal day 8 (end of HF morphogenesis) and isolated skin samples at postnatal days 17, 19, 21, 28, 32, and 49, which are critical time points in the HF cycle. Moreover, HF cycling was induced by depilation in conditional EGFR mice after several weeks of tamoxifen treatment and skin samples were collected at different time points. The histomorphometric analysis of these skin biopsies is currently being performed and will bring new insights if and when EGFR signaling functions as a biological switch in the hair growth cycle.

All together, the results obtained so far confirm that EGFR signaling delays HF morphogenesis as well as the entry into hair follicle cycling. Moreover, EGFR expression is essential for the development of a proper hair shaft and orchestrates the orientation and distribution of hair follicles.

5.1.5 EGFR mutant mice display strong inflammation in the skin

Histological analysis of skin samples revealed a strong infiltrate of inflammatory cells within the dermis of EGFR mutant skin (Fig. 12K,L,P). Giemsa staining of skin sections showed that the number of mast cells was increased in $EGFR^{\Delta ep}$ skin at distinct time points (P1, P10, P19 and P21; Fig.14A). Interestingly, these mast cells were highly degranulated ($\geq$ 8 granuli), which indicates an activated state of these cells. So far the role of mast cells in the HF immune system is not completely understood. However, they could be involved in the regulation of HF cycling. To further characterize the inflammatory infiltrate in EGFR mutant skin I isolated epidermal and dermal cells as well as cells from thymus and skin-draining lymph nodes. Flow-cytometric analysis of these cells revealed that 40% of keratinocytes isolated from $EGFR^{\Delta ep}$ skin express high levels of MHC II on

the cell surface, whereas only a negligible number of keratinocytes isolated from wild-type mice was MHC II^+ (Fig. 14D). This indicates that EGFR mutant keratinocytes are in an activated state. Moreover, the percentage of $CD45^+$ hematopoietic cells in the epidermal compartment of mutants was 5 times more compared to wild-type skin (Fig. 14E). In addition, the number of MHC II^+ $CD45^+$ cells, comprising mainly Langerhans cells, was significantly increased (Fig. 14F). Also, the number of T-cells was higher in $EGFR^{\Delta ep}$ epidermis (Fig. 14G). Interestingly, reduced numbers of resident γδT-cells ($CD3\varepsilon^{high}$) were detected in EGFR mutant epidermis, suggesting that the increase in T-cells was due to newly infiltrated $CD3\varepsilon^{low}$ αβT-cells (Fig. 14H and data not shown). Within this population a significantly increased number of cytotoxic T-cells ($CD8\alpha^+$) could also be detected (Fig. 14I).

Similarly, in the dermal compartment of EGFR mutant skin a large number of activated, MHC II^+ fibroblasts and endothelial cells (ECs) was detected (Fig. 15A). Accordingly, the percentage of hematopoietic cells was significantly increased in the dermis of $EGFR^{\Delta ep}$ mice compared to the dermis of littermate controls, and out of these cells one third consisted of Dendritic Cells (DCs; MHC II^+ $CD45^+$) like in wild-type mice (Fig. 15B,C). Interestingly, a large number of granulocytes had infiltrated into the dermis of $EGFR^{\Delta ep}$ mice (Fig. 15D). The percentage of T-cells was only slightly increased in the dermal compartment of $EGFR^{\Delta ep}$ skin (Fig. 15E), and more $CD8\alpha^+$ cells were detected (Fig. 15F). Since the K5 promoter is also active in thymic epithelial cells I was also interested whether EGFR deletion in these cells would affect T-cell development. The analysis of the thymus and skin-draining lymph nodes did, however, not reveal prominent differences in the percentages of DCs, T-cells, granulocytes and macrophages between wild-type and $EGFR^{\Delta ep}$ mice. Surprisingly, the number of $FoxP3^+$ $CD25^+$ regulatory T-cells was elevated in skin-draining lymph nodes of $EGFR^{\Delta ep}$ mice (Fig. 15G).

5.1.6 Hair follicles do not affect skin inflammation in EGFR mutant mice

In mice humanized for the EGFR ($hEGFR^{KI/KI}$), which show reduced expression of the EGFR in the skin, the hair follicles remain stuck at the anagen/catagen transition of the first hair cycle leading to loss/degeneration of the hair follicles.

These alterations lead to severe dermal fibrosis with massive infiltration of inflammatory cells and the mice are bald by the age of 3-4 months (Sibilia et al., 2003). It is still unclear whether the inflammatory cells are attracted to the dermis as a consequence of hair follicle degeneration or whether deletion of EGFR in the epidermis perturbs the dermal microenvironment such that inflammatory cells are recruited to the skin and attack the hair follicles leading to their destruction. To clarify these issues, I started to cross $EGFR^{\Delta ep}$ mice and $hEGFR^{KI/KI}$ mice with hairless (hr/hr) mice or immunodeficient $Rag2^{-/-}$ mice. Hairless hr/hr mutant mice carry a mutation in the hairless (hr) gene and progressively loose hair becoming completely bald 3-4 weeks after birth (Brancaz et al., 2004). No immunological defects or skin inflammation have been described for these mice. In contrast, $Rag2^{-/-}$ mice develop normal hair but are immunodeficient due to a lack of mature T- and B-cells (Shinkai et al., 1992). Histological and flow-cytometric analysis of skin sections of double mutant $EGFR^{\Delta ep}$ hr/hr and $hEGFR^{KI/KI}$ hr/hr as well as $EGFR^{\Delta ep}$ $Rag2^{-/-}$ and $hEGFR^{KI/KI}$ $Rag2^{-/-}$ mice will allow me to discriminate whether the skin inflammation found in EGFR mutant mice is the cause or the consequence of the severe hair follicle degradation. Unfortunately, I have had only the opportunity to analyze $EGFR^{\Delta ep}$ hr/hr mice so far since the breeding performance was poor. The analysis of 3-week old double mutants by flow cytometry revealed that the quality and quantity of the inflammatory infiltrate was comparable to single $EGFR^{\Delta ep}$ mice (Fig. 14D-I; Fig. 15A-G). However, at the age of 3 weeks $EGFR^{\Delta ep}$ hr/hr still harbored many hair follicles in the skin even though their $EGFR^{f/f}$ hr/hr littermate controls were already bald. This might be due to the delay in HF morphogenesis and cycling in $EGFR^{\Delta ep}$ mice.

Interestingly, the very few surviving $EGFR^{\Delta ep}$ and $EGFR^{\Delta ep}$ hr/hr mice which were almost bald at the age of 4 months showed a surprising difference: while macroscopically the skin of the $EGFR^{\Delta ep}$ mouse was strongly inflamed and necrotic, the skin of the $EGFR^{\Delta ep}$ hr/hr looked overtly normal and did not display any necrotic lesions (Fig. 14B,C). However, a detailed flow-cytometric analysis of the cells isolated from these mice and their littermate controls revealed that inflammation in the skin of 4 months old $EGFR^{\Delta ep}$ hr/hr mice is comparable to $EGFR^{\Delta ep}$ mice and resembles the inflammatory phenotype of 3.5 week-old mice (Fig. 14D-I; Fig. 15A-G and data not shown). Thus, we can conclude that the

strong inflammation found in EGFR deficient skin does not result from degenerating hair follicles.

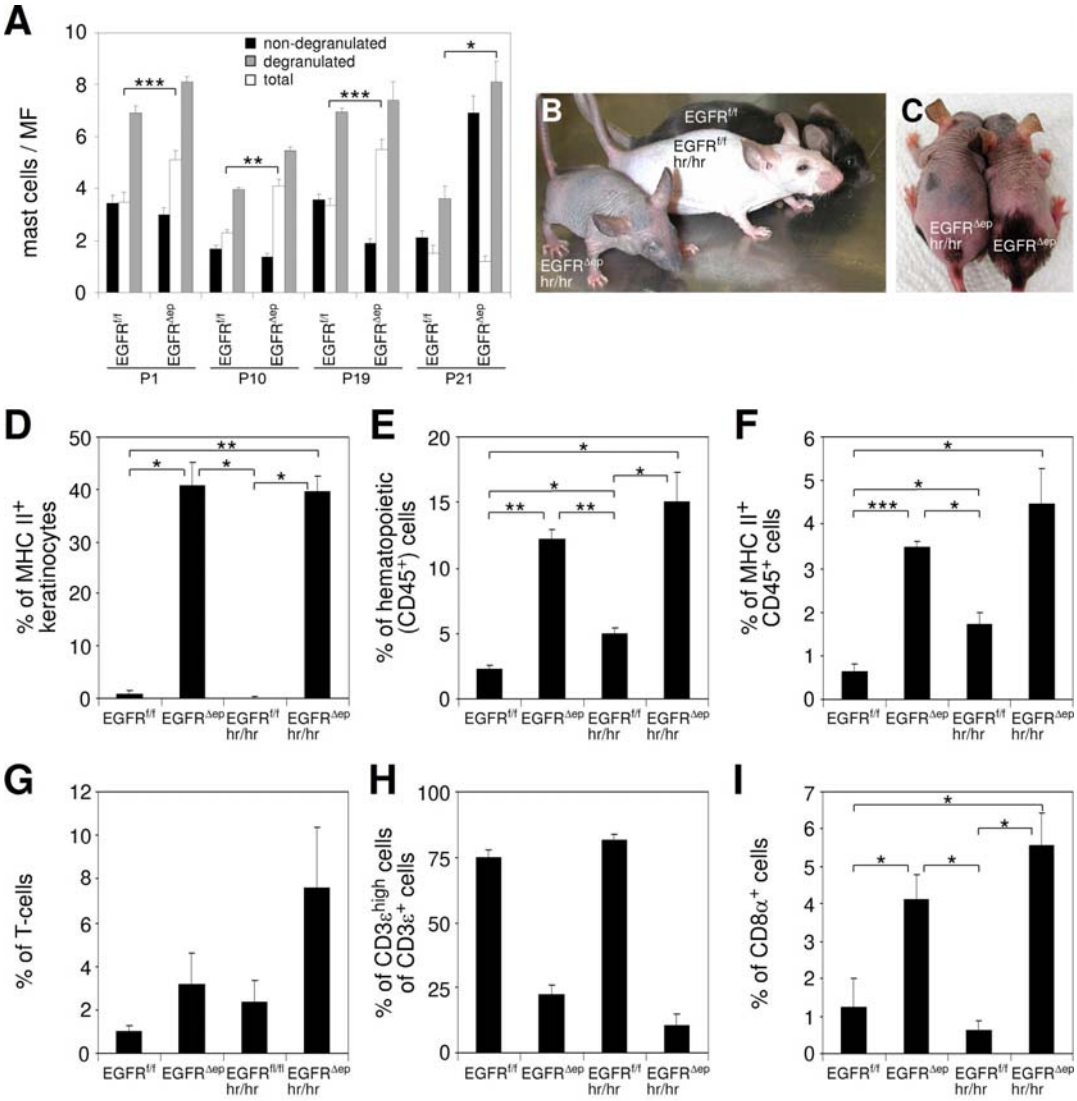

Figure 14. Lack of EGFR expression results in inflammation of the skin. (A) Quantitative analysis of Giemsa staining of skin samples from EGFR$^{\Delta ep}$ and wild-type mice isolated at various time points after birth reveals an increased number of (activated) mast cells in EGFR mutant skin (degranulated ≥ 8 granuli). Data represent mean ± SEM (n = 3). * p≤0.05; ** p≤0.005; *** p≤0.0005. **(B,C)** Phenotype of 4 months old EGFR$^{\Delta ep}$ and EGFR$^{\Delta ep}$ hr/hr and control mice. Note that the back skin of EGFR$^{\Delta ep}$ hr/hr mice looks overtly normal, while the skin of EGFR$^{\Delta ep}$ mice displays necrotic lesions. **(D-I)** Flow cytometric analysis of epidermal cells isolated from mice with the indicated genotypes. Data represent mean ± SEM (n = 3). * p≤0.05; ** p≤0.005; *** p≤0.0005.

In summary, these results suggest that the lack of EGFR expression in the epidermis leads to a strong inflammation in the skin. It will be interesting to unravel if EGFR mutant HF loose their immune privilege by aberrant MHC I expression in the outer root sheath of HF.

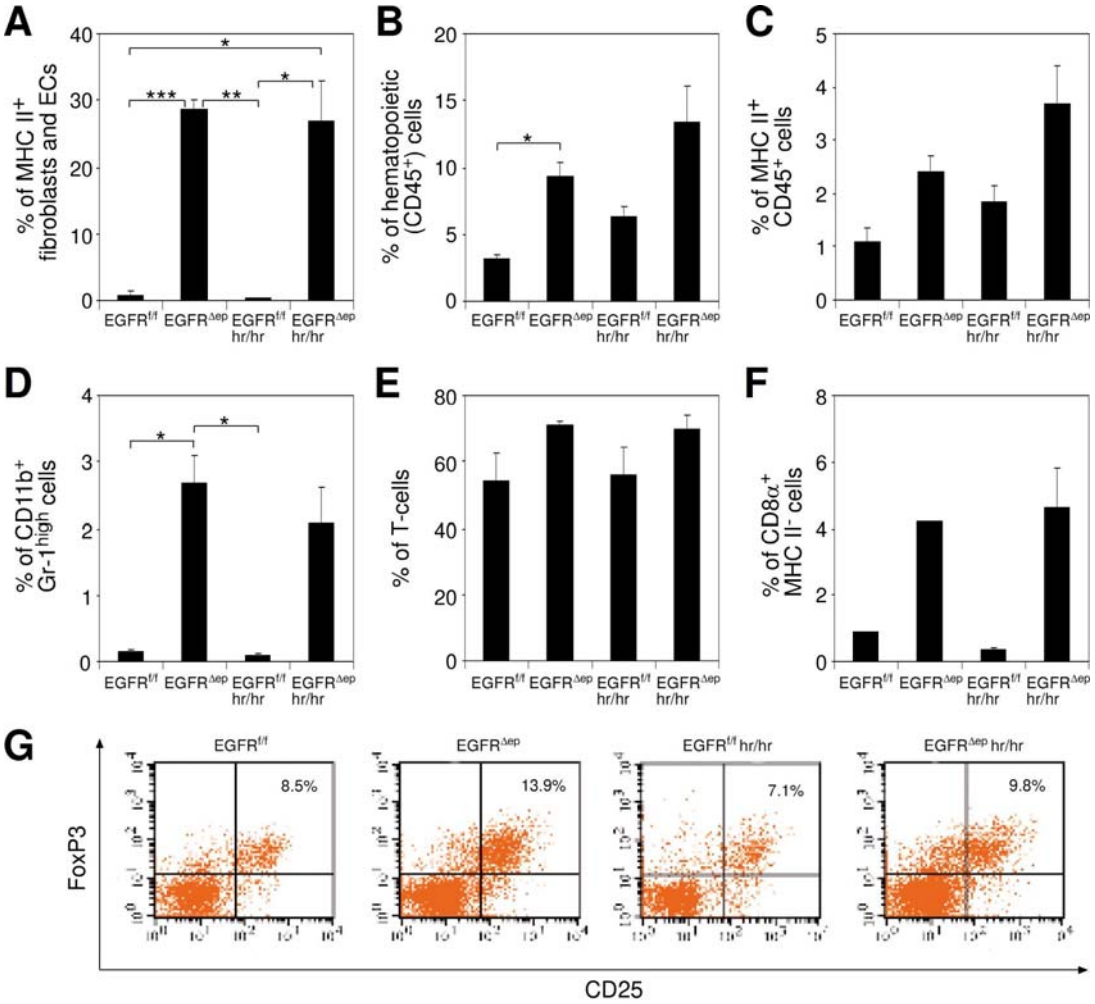

Figure 15. Lack of EGFR expression results in inflammation of the skin. (A-F) Flow cytometric analysis of dermal cells isolated from mice with the indicated genotypes. Data represent mean ± SEM (n = 3). * p≤0.05; ** p≤0.005; *** p≤0.0005**. (G)** Flow cytometric analysis of cells isolated from skin-draining lymph nodes of mice with the indicated genotypes. Cells were gated on CD4.

5.1.7 Wound healing is delayed in mice lacking EGFR expression in the skin

Because of the short viability of $EGFR^{-/-}$ mice the function of EGFR in the complex processes of wound healing could not be addressed in these mice. However, incisional wounds in EGFR deficient skin grafts have implicated a role of EGFR in wound repair. In order to investigate the function of EGFR in wound healing I placed full thickness punch wounds at the back of adult tamoxifen-treated $EGFR^{\Delta epER}$ mice and of $hEGFR^{KI/KI}$ mice, as well as of 3.5-week old $EGFR^{\Delta ep}$ mice and the respective littermate controls. Analysis of the wounds at

different time points after injury revealed that in all three experiments the lack of EGFR expression in the skin significantly delayed wound closure (Fig. 16A-C).

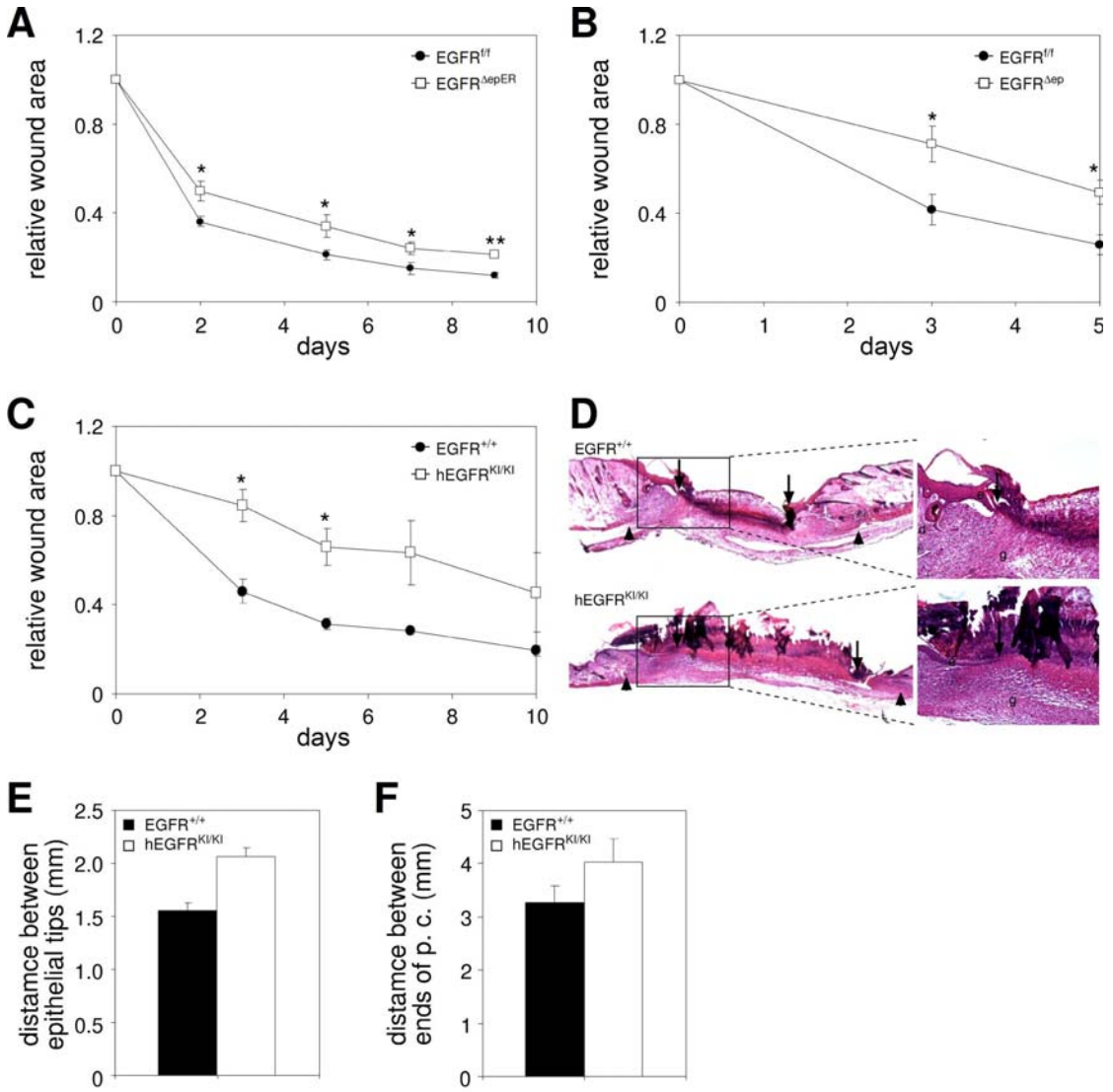

Figure 16. Lack of EGFR affects wound healing. (A-C) Relative wound area of full thickness punch wounds in mice of the indicated genotypes at different time points upon injury. Data represent mean ± SEM (n = 5-9). * p≤0.05; ** p≤0.005**. (D)** H&E staining of sections from full thickness punch wounds of a hEGFR$^{KI/KI}$ mouse and a littermate control. Arrows point to the tips of epithelial tongues, arrow hards indicate wound edges of *panniculus carnosus.* d – dermis; e – epidermis; g – granulation tissue. **(E,F)** Distance between epithelial tips (E) and edges of *panniculus carnosus* (F) in wounds of hEGFR$^{KI/KI}$ mice and littermate controls (n = 2)

Preliminary data from the histological analysis of Hematoxylin and Eosin (H&E) stained sections of wounds isolated at day 5 upon injury from hEGFR$^{KI/KI}$ and their wild-type littermate controls (Fig. 16D) revealed that the lack of EGFR affects reepithelialization and wound contraction. The distance between the

edges of the wounds, and the distance between the tips of the epithelial tongues as well was longer in wounds from hEGFR$^{KI/KI}$ compared to those of controls (Fig. 16E,F). These results suggest that the lack of EGFR in the epidermis delays the wound healing process by affecting both reepithelialization and wound contraction. However, these are only preliminary data and wound sections of more biopsies isolated at different time points need to be analyzed. Moreover, it should be examined if EGFR affects keratinocyte proliferation and wound angiogenesis, and if EGFR is involved in the recruitment of inflammatory cells to the wound site.

5.1.8 Discussion and Outlook

There is no doubt that the EGFR signaling pathway belongs to the most complex networks in an organism. Seven ligands bind with different affinities to 4 different receptors of the EGFR family, which may homo- or heterodimerize upon ligand binding, thus, allowing multiple combinatorial possibilities of signaling. Besides, differential expression of ligand precursors and their activating proteinases complicate this multifaceted system even more. Therefore, it is conceptional that even though EGFR signaling has been in the focus of researchers for several decades, we are just beginning to understand the complex cellular processes controlled by EGFR. The analysis of mice genetically modified for the expression of EGFR or its ligands have disclosed that the EGFR regulates diverse processes in different tissues and cells. For instance, EGFR signaling was shown to be an important survival signal in epidermal tumors and cortical astrocytes, whereas in chondrocytes and osteoblasts EGFR seems to affect differentiation, and in hepatocytes proliferation and cell-cycle entry upon tissue injury (Sibilia et al., 2007). The mostly mild phenotypes of mice lacking a single EGFR ligand propose redundancy as the evolutionary strategy to assure proper development and tissue homeostasis even if a single ligand is missing. Only HB-EGF was shown to be essential for heart development and wound reepithelialization (Schneider et al., 2008b).

A multitude of studies in mice lacking the EGFR or its ligands or overexpressing them have demonstrated that the EGFR signaling network is crucial in epithelia. The results obtained in this study confirm once more that EGFR is an important player in the development and homeostasis of the skin and its appendages. What was unexpected and surprising, though, was the fact that epidermis-specific deletion of EGFR is lethal. Since the K5 promoter is also active in epithelial tissues of the intestine the early postnatal lethality of $EGFR^{\Delta ep}$ mice might be due to EGFR deletion in the GI-tract and, thus, a functionally impaired intestine. However, unpublished results from our laboratory revealed that mice lacking EGFR expression only in intestinal epithelia ($EGFR^{f/f}$ Villin-Cre = $EGFR^{\Delta int}$) (Egan et al., 2004) are viable and do not have any conspicuous phenotype (Rost et al., unpublished). Evidence that EGFR null mice might die because of epithelial defects already came from the analysis of EGFR null mice expressing a constitutive active form of the Ras activator son of sevenless (K5-SOS) in the basal layers of the epidermis, which prolonged the lifespan of EGFR knock-out mice to about 6 months (Sibilia et al., 2000). Interestingly, tamoxifen-induced deletion of EGFR in the epidermis of neonatal mice was lethal, too, whereas EGFR deletion in adult mice did not affect the survival of the mice but led to mild derangements of hair follicles only. We could show that the formation of the pivotal skin barrier was delayed in EGFR mutant mice, but neonatal EGFR mutants did not have any defects in the skin barrier. However, we cannot exclude that the skin becomes permeable at a later time point, since the morphology of EGFR mutant skin gets worse with time. Therefore, skin permeability assays need to be performed in older animals. Thus, these results suggest that EGFR is indispensable during embryonic development of the skin and in the first postnatal days to assure later survival. It is possible that EGFR deficient epidermis does not secrete important systemically acting factors, which might essentially affect overall survival. Preliminary data have revealed that high levels of TNFα are found in the serum of both $EGFR^{-/-}$ and $EGFR^{\Delta ep}$ mice compared to their littermate controls. Sustained activation of TNFα signalling has been implicated in the pathogenesis of several human diseases, and was recapitulated in TNFα transgenic mice (Aggarwal, 2003; Butler et al., 1997; Kontoyiannis et al., 1999). It is established that high levels of TNFα induce cachexia, which leads to an increased energy consumption and

subsequently to death. However, breeding of $EGFR^{-/-}$ or $EGFR^{\Delta ep}$ mice to TNFR knock-out mice did not rescue the early lethality of EGFR mutant mice (data not shown), thus, excluding that excess TNFα might be responsible for the lethal phenotype.

Importantly, the analysis of full-thickness punch wounds in different EGFR mutant mice revealed that wound repair is significantly delayed upon EGFR deletion, but EGFR is not indispensable for wound healing. Preliminary histological investigations of wound biopsies have demonstrated that EGFR signaling accelerates both wound reepithelialization and wound contraction. This observation is consistent with the study of incisional wounds in EGFR null skin-grafts, where the EGFR was shown to be implicated in proliferation, migration, wound contraction, angiogenesis, and inflammation (Repertinger et al., 2004). The effect of EGFR signaling on keratinocyte proliferation, migration, on the myofibroblast compartment, on wound angiogenesis and induction of inflammatory responses need to be further examined. Moreover, wound repair (and skin and HF development) depends on epidermal stem cells. Therefore it would be interesting to investigate if the lack of EGFR affects the epidermal stem cell compartment. Label-retaining-cell assays in $hEGFR^{KI/KI}$ and $EGFR^{\Delta epER}$ mice may be very helpful to address this aspect.

Furthermore, I could show that both HF morphogenesis and the entry into hair follicle cycling is delayed in $EGFR^{\Delta ep}$ mice. In addition, our results confirm that EGFR expression is essential for the development of a proper hair shaft and orchestrates the orientation and distribution of hair follicles. The analysis of skin biopsies from mice, in which EGFR was deleted after HF morphogenesis, and the study of depilation-induced HF cycle induction in $EGFR^{\Delta epER}$ mice will allow us to unravel whether EGFR functions as a biological switch to drive HF cycling.

A very interesting finding was that EGFR mutant skin is strongly inflamed. Both the epidermis and the dermis displayed signs of inflammation: strong MHC II expression was found both on keratinocytes, and dermal fibroblasts and endothelial cells. The number of hematopoietic cells was increased several-fold in both skin compartments of $EGFR^{\Delta ep}$ mice compared to EGFR wild-type controls. A large number of Langerhans cells was found in the epidermis of EGFR deficient skin, which was accompanied by a high infiltrate of αβT-cells. The dermis was infiltrated by DCs, granulocytes and mast cells, and - similar to

the epidermis – by cytotoxic CD8α^{+} T-cells. There is increasing evidence that the EGFR pathway has an important impact on the inflammatory reactions of the skin, with the effort to enhance innate immune responses on the one hand, and to prevent over-activation of the pro-inflammatory function of epidermal cells on the other hand (Pastore et al., 2008). Keratinocytes express various Toll-like receptors (TLRs), which seem to be the primary sensors of innate immunity. It was shown that TGFα induces expression of TLR5 and TLR9 in keratinocytes and cooperates with these receptors to upregulate the expression of anti-microbial peptides (Pastore et al., 2008). Furthermore, accelerated expression of these peptides following skin injury depends on EGFR signaling rather than the presence of microbial components (Sorensen et al., 2006). A recent study showed that anti-microbial peptides stimulate keratinocyte proliferation and migration in an EGFR-dependent manner, and induce *de novo* expression of cytokines and T-cell chemoattractants (Niyonsaba et al., 2007), thereby promoting an adaptive immune response in the skin. Chronic inflammatory skin diseases, such as psoriasis, atopic dermatitis or allergic contact dermatitis are often accompanied by epidermal hyperplasia mediated by leukocyte-derived cytokines like TNFα or interferon γ (IFNγ), which are potent inducers of EGFR and its ligands and initiate a program leading to enhanced expression of inflammatory mediators (Pastore et al., 2008). Importantly, mice expressing a dominant-negative EGFR and also hEGFR$^{KI/KI}$ mice expressing very low levels of (human) EGFR in the skin display severe HF derangements, and hair follicles are eventually degraded, which is accompanied by a large infiltrate of inflammatory cells (Hansen et al., 1997; Murillas et al., 1995; Sibilia et al., 2003). If the inflammation found in EGFR deficient skin is the consequence of HF degeneration or whether EGFR deletion in the epidermis disrupts the dermal microenvironment such that inflammatory cells are recruited to the skin and attack the hair follicles leading to their degeneration, still needs to be elucidated. EGFR$^{\Delta ep}$ hr/hr and hEGFR$^{KI/KI}$ hr/hr as well as EGFR$^{\Delta ep}$ Rag2$^{-/-}$ and hEGFR$^{KI/KI}$ Rag2$^{-/-}$ mice double mutants will be of great help for these investigations. Moreover, it should be clarified whether HF of EGFR mutant skin loose their immune privilege by aberrant MHC I expression in the outer root sheath of HF.

The need for a better understanding of the impact of EGFR signaling on skin inflammation has become urgent since anti-EGFR therapies for epithelial cancers have been introduced. A common, severe, and often therapy-limiting undesired treatment effect of anti-EGFR monoclonal antibodies and EGFR tyrosine kinase inhibitors, which are oncologically very attractive drugs, is the development of inflammatory rashes and abnormal hair growth. However, in many cancer patients the presence of cutaneous side effects is positively correlated with tumor regression or even survival (Hynes and Lane, 2005; Lacouture, 2006; Perez-Soler and Saltz, 2005). Thus, the skin offers an excellent read-out system for studying the complex EGFR signaling network, and for evaluating the efficacy of anti-EGFR therapies. Moreover, it raises the question whether the cutaneous side effects are even beneficial for the clinical outcome of EGFR-antagonistic treatment.

5.2 MANUSCRIPT: Autocrine VEGF signaling synergizes with EGFR in tumor cells to promote epithelial cancer development

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SUMMARY

It is established that VEGF produced by tumor cells acts on neighboring endothelial cells to promote angiogenesis and tumor growth. Here we demonstrate that in a K5-SOS-dependent mouse skin tumor model, autocrine VEGF is required for epithelial tumor cell proliferation in a cell-autonomous manner. VEGF is upregulated in SOS-expressing tumors and genetic deletion specifically in epidermal cells delays tumor development. Surprisingly, complete inhibition of tumor development was found in the absence of VEGF in a mutant EGFR background, demonstrating that VEGFR and EGFR signaling synergize in neoplastic cells to promote tumor growth. Similar results were obtained with therapeutics inhibiting EGFR and VEGFR. Mechanistically, K5-SOS upregulates VEGF and its receptors Flt1 and Neuropilin-1 in an Erk-dependent manner thereby activating an autocrine proliferation loop, whereas EGFR acts as a survival factor for tumor cells. Moreover, Flt1 is expressed in the majority of human squamous cell carcinomas (SCC) and its inhibition in human SCC cell lines impairs proliferation emphasizing the medical relevance of our findings. Thus, in addition to regulating angiogenesis, VEGF has to be considered as a potent growth factor for epidermal tumors.

INTRODUCTION

The epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases (RTK) includes ErbB1/EGFR, ErbB2/Neu, ErbB3 and ErbB4 and is activated by ligand-dependent homo- or heterodimerisation (Schlessinger, 2002). Genetic ablation experiments in mice revealed the importance of EGFR for the development of different organs like brain, bone, heart and several epithelial tissues (Sibilia et al., 2007). EGFR overexpression and mutations have been detected at high frequency in tumors of epithelial and glial origin, the same cell types affected also in mice lacking the EGFR (Olayioye et al., 2000; Sibilia et al., 2007). The cellular processes controlled by EGFR are complex and context-dependent. In the liver, the EGFR seems to control hepatocyte proliferation during liver regeneration, whereas in astrocytes it seems to act as a survival signal (Natarajan et al., 2007; Wagner et al., 2006). We have also shown that transgenic mice expressing a constitutively active form of the Ras activator Son of Sevenless from the keratin 5 promoter (K5-SOS mice) develop spontaneous skin papillomas at 100% penetrance in a wild-type EGFR background. However, in a hypomorphic $EGFR^{wa2/wa2}$ or null background, K5-SOS-dependent tumor formation is severely impaired (Sibilia et al., 2000). In these mice the EGFR provides an essential survival signal to tumor cells by activating the anti-apoptotic Akt pathway (Sibilia et al., 2000).

Tumors and metastases usually arise as small avascular masses which subsequently induce neovascularization in order to acquire nutrients for continued growth and metastatic spread. This angiogenic switch is induced by angiogenic factors secreted by tumor cells (Bergers and Benjamin, 2003; Hirakawa et al., 2005). The vascular endothelial growth factor A (VEGF) is one such key regulator of tumor angiogenesis and the role of the VEGF family in the control of angiogenesis has been intensively investigated for more than a decade (Ferrara, 2002). VEGF is produced as different isoforms resulting from alternative exon splicing: 3 isoforms in mice ($VEGF_{120}$, $VEGF_{164}$, $VEGF_{188}$) and 9 transcripts in humans (Ferrara et al., 2003; Takahashi and

Shibuya, 2005). While in mice $VEGF_{120}$ diffuses freely in the surrounding extracellular matrix, the other isoforms show increased binding to the heparin-rich extracellular matrix (Carmeliet and Collen, 1999). VEGF binds to two related RTKs, VEGFR1/Flt1 and VEGFR2/KDR/Flk1, both of which are primarily expressed on vascular endothelial cells and upregulated during angiogenic processes. In addition, VEGF interacts with a family of coreceptors, the neuropilins (Nrp), which were identified to be involved in axon guidance and are now known to enhance VEGFR signaling (Ferrara et al., 2003).

VEGF has been shown to be a survival factor for endothelial cells and to play an essential role in embryonic vasculogenesis and angiogenesis (Gerber et al., 1999; Lee et al., 2007a). Disruption of one single VEGF allele is embryonic lethal due to abnormal vasculature (Carmeliet et al., 1996; Ferrara et al., 1996). Similarly, $Flt1^{-/-}$ and $Flk1^{-/-}$ mice die *in utero* due to early defects in the development of hematopoietic and endothelial cells, respectively (Fong et al., 1995; Shalaby et al., 1995). Conditional deletion of VEGF within the first postnatal weeks in various organs such as kidney, liver, spleen, bone marrow, heart and brain results in profound vascular anomalies and lethality. VEGF inactivation in older animals is much less traumatic suggesting that VEGF does not have a continuous maintenance function for the adult vasculature (Gerber et al., 1999). However, genetic deletion of VEGF in the endothelial lineage leads to progressive endothelial degeneration and sudden death in mutant mice by 25 weeks of age suggesting that autocrine VEGF signaling is required for vascular homeostasis (Lee et al., 2007a). Interestingly, mice overexpressing $VEGF_{120}$ in the suprabasal compartment of the epidermis via the keratin 6 promoter displayed skin swelling, erythema and edema subsequently leading to the disruption of skin architecture and early postnatal lethality (Larcher et al., 1998). It is therefore evident that VEGF is a potent vascular regulator and that its dosage must be exquisitely regulated in a spatial, temporal and quantitative manner to avoid vascular disorders.

VEGF overexpression is found in many human and murine tumors such as those of epithelial origin (Dvorak, 2002; Larcher et al., 1996). VEGF upregulation in tumors is

linked to hypoxia and/or overexpression of a variety of oncogenes including mutant ras, erbB2/Her2, bcr-abl and activated EGFR. The classical role for VEGF produced by tumor cells is that it acts on neighboring endothelial cells thereby promoting tumor vascularization (Bergers and Benjamin, 2003; Ferrara et al., 2003; Ferrara and Kerbel, 2005). For example during two-stage skin chemical carcinogenesis in mice it was shown that EGFR signaling is responsible for Ha-ras-dependent VEGF upregulation and induction of the angiogenic switch necessary for tumor growth (Casanova et al., 2002). In this model, expression of dominant negative EGFR in tumors could abolish blood vessel remodeling suggesting that a severe reduction of VEGF expression is the critical event responsible for angiogenesis and tumor growth suppression. Accordingly, epidermis-specific deletion of VEGF impaired carcinogen-induced papilloma formation (Rossiter et al., 2004). Recent reports demonstrated expression of Flt1 and Flk1 as well as Nrp1 on tumor cells (Chung et al., 2006; Fakhari et al., 2002; Lee et al., 2007b; Parikh et al., 2004). Although *in vitro* it could be shown that VEGFR signaling can mediate intracrine survival of tumor cell lines (Lee et al., 2007b), the expression and function of VEGFRs in tumors *in vivo* remains controversial.

By employing K5-SOS transgenic mice, we provide the first demonstration that VEGFR signaling is cell-autonomously required in skin tumor cells to stimulate their proliferation in an autocrine manner and that VEGFR and EGFR signaling synergize in neoplastic cells to promote tumor growth. We show that epidermal tumor cells of K5-SOS transgenic mice express high levels of VEGF and its receptors Flt1 and Nrp1 in an Erk-dependent manner. Genetic deletion of VEGF in the epidermis of K5-SOS transgenic mice delays K5-SOS-dependent skin papilloma formation in a wild-type EGFR background and completely inhibits tumor development in a mutant EGFR background. Most importantly, Flt1 is upregulated in the majority of human squamous cell carcinomas (SCC), a common skin neoplasm frequently harboring activating Ras mutations, thereby highlighting the relevance of the murine findings for human epithelial tumor development.

RESULTS

Epidermal-specific VEGF deletion delays K5-SOS-dependent tumor development

Since EGFR signaling can induce expression of VEGF in keratinocytes (Casanova et al., 2002), we analyzed whether VEGF expression was affected in K5-SOS transgenic epidermis. VEGF mRNA expression and protein secretion could be detected in control keratinocytes, but was reduced by 40% in $EGFR^{wa2/wa2}$ and $EGFR^{-/-}$ keratinocytes (Fig. 1A,B). Interestingly, K5-SOS expression significantly increased the levels of VEGF mRNA and protein secretion in both $EGFR^{wa2/+}$ as well as $EGFR^{wa2/wa2}$ keratinocytes (Fig.1A,B). These results demonstrate that EGFR and SOS positively control VEGF expression.

To investigate the effect of VEGF deletion during SOS-dependent skin tumor development, K5-SOS transgenic mice of EGFR wild-type and hypomorphic background were crossed with $VEGF^{f/f}$ mice (Gerber et al., 1999), and further bred with a K5-Cre transgenic line (Tarutani et al., 1997) to delete VEGF in basal epidermal cells ($VEGF^{\Delta ep}$ mice). Cre-mediated recombination of the VEGF gene was very efficient in epidermal cells and transcription of VEGF mRNA as well as secretion of VEGF protein could not be detected in keratinocytes isolated from $VEGF^{\Delta ep}$ mice (Fig. 1B, Suppl. Fig. 1A,B). In contrast, VEGF expression in skin endothelial cells was not affected in these mice (Suppl. Fig. 1C,D). Deletion of both VEGF alleles in the epidermis significantly delayed the development of skin papillomas which started to appear only after 1.5 months (Fig. 1C). The average tumor volume was also significantly smaller in K5-SOS tumors lacking VEGF (Fig. 1D). Even after 3 months only 18% of the tumors exceeded this volume (data not shown). Deletion of only one VEGF allele did not affect the onset of tumor development and the average tumor volume (data not shown). These results indicate that VEGF deletion in epidermal cells significantly impairs K5-SOS-dependent skin tumor development.

No tumor development in the absence of EGFR and VEGF

K5-SOS-dependent skin tumor development is severely impaired in the hypomorphic $EGFR^{wa2/wa2}$ background and papillomas develop very late and never reach the size of the tumors observed in an EGFR wild-type background (Sibilia et al., 2000) Fig. 1E,F). Inactivation of VEGF in the epidermis had a dramatic effect on K5-SOS-dependent tumor growth. When both VEGF alleles were deleted papilloma development was completely inhibited and 100% of $EGFR^{wa2/wa2}$ $VEGF^{\Delta ep}$ K5-SOS mice remained tumor free for more than 1 year (Fig. 1E). Tumor incidence and the average tumor volume in the absence of one VEGF allele was slightly delayed compared to $EGFR^{wa2/wa2}$ K5-SOS mice (Fig. 1E,F). These results show that no tumors develop in the absence of EGFR and VEGF signaling in K5-SOS transgenic mice.

Wounding can induce and accelerate tumor formation in $EGFR^{wa2/wa2}$ K5-SOS transgenic mice and skin papillomas start to appear two weeks after wounding (Fig. 1G,H). To investigate if VEGF was responsible for wounding-induced tumors, small incisions were applied at the tip of one ear of $EGFR^{wa2/wa2}$ K5-SOS mice lacking one or both VEGF alleles. In $EGFR^{wa2/wa2}$ $VEGF^{f/+}$ K5-Cre K5-SOS mice the increase in tumor volume was initially comparable to $EGFR^{wa2/wa2}$ K5-SOS mice (Fig. 1G). However, from day 20 onwards tumor growth was delayed in the presence of only one functional VEGF allele and after 35 days tumors were significantly smaller (Fig. 1G-I). In contrast, after 35 days no tumors had developed in $EGFR^{wa2/wa2}$ K5-SOS mice lacking both VEGF alleles and these mice remained tumor free for the remaining 12 months they were under observation (Fig. 1G,J). These results demonstrate that in a mutant EGFR background K5-SOS-dependent tumor growth after wounding is completely inhibited in mice lacking both VEGF alleles suggesting a synergistic effect of EGFR and VEGF signaling in tumor development.

VEGF deletion dramatically affects tumor cell proliferation and blood vessel density

We next examined whether the absence of VEGF affects the vascularization, differentiation or proliferation of spontaneous or wounding-induced skin papillomas. As previously described, papillomas of $EGFR^{wa2/wa2}$ K5-SOS mice were more differentiated as evidenced by the higher number of K1 positive cells and this was not further affected by the absence of VEGF (Suppl. Fig. 2A, (Sibilia et al., 2000)). However, the average vessel area was much lower in skin papillomas from $EGFR^{wa2/+}$ $VEGF^{\Delta ep}$ K5-SOS mice compared to the respective control tumors and the average diameter of blood vessels was smaller (Fig. 2A,B). Deletion of only one VEGF allele did not affect vascularization in K5-SOS tumors from EGFR mutants (Fig. 2A,B).

Interestingly, the absence of VEGF did not only reduce tumor angiogenesis but also dramatically decreased the number of proliferating tumor cells (Fig. 2A,C). Five times less Ki67 positive cells were detectable in skin papillomas derived from $EGFR^{wa2/+}$ $VEGF^{\Delta ep}$ K5-SOS mice than in biopsies from $EGFR^{wa2/+}$ K5-SOS controls. Furthermore, already the deletion of one VEGF allele resulted in significantly reduced cell proliferation in wounding-induced tumors of $EGFR^{wa2/wa2}$ K5-SOS mice (Fig. 2C). Basal cell proliferation was also significantly reduced in healthy normal skin of EGFR wild-type and hypomorphic mice upon VEGF deletion (Suppl. Fig. 1E). These results suggest that VEGF controls epidermal cell proliferation, which in combination with reduced angiogenesis leads to the complete inhibition of tumor formation in $EGFR^{wa2/wa2}$ $VEGF^{\Delta ep}$ K5-SOS mice.

EGFR signaling is required in epidermal cells to induce tumor formation

In $EGFR^{wa2/wa2}$ mice the EGFR is mutated in all cells including endothelial cells. To investigate whether functional EGFR signaling is required in a cell-autonomous manner in epidermal cells for tumor induction, we employed mice carrying conditional EGFR alleles ($EGFR^{f/f}$) to delete the EGFR exclusively in the epidermis of K5-SOS $VEGF^{f/f}$

mice (Natarajan et al., 2007). For temporal control of EGFR and VEGF deletion we employed the tamoxifen-inducible K5-CreER^{T} transgenic line (Indra et al., 1999) allowing us to specifically delete the EGFR (EGFR$^{\Delta epER}$) and VEGF (VEGF$^{\Delta epER}$) in epidermal cells at different stages of tumor development.

EGFR$^{f/f}$ VEGF$^{f/f}$ K5-CreER^{T} K5-SOS and as comparison EGFR$^{wa2/wa2}$ VEGF$^{f/f}$ K5-CreER^{T} K5-SOS mice with their respective littermate controls were injected with tamoxifen for 2 weeks to delete EGFR and/or VEGF and then tumor growth was induced by a small incision at the ear tip as outlined in Figure 3A (preventive trial). While tumor onset occurred approximately two weeks after wounding in all five groups, tumor volume was significantly reduced in mice lacking VEGF in the epidermis both in an EGFR$^{wa2/wa2}$ and EGFR$^{\Delta epER}$ background (Fig. 3A). Interestingly, deletion of only one VEGF allele in EGFR$^{\Delta epER}$ mice was enough to severely reduce tumor growth. This was not observed in an EGFR$^{wa2/wa2}$ background likely because the hypomorphic EGFR can still signal, even though at reduced levels compared to a wild-type EGFR. Immunofluoresent staining for EGFR on papilloma sections confirmed the absence of EGFR in EGFR$^{\Delta epER}$ tumors (Fig. 3C). Moreover, no or only extremely low levels of VEGF could be detected in VEGF$^{\Delta epER}$ keratinocytes (Fig. 3D). These results demonstrate that the lack of EGFR expression in the epidermis is sufficient to inhibit K5-SOS-dependent skin tumor formation in the absence of VEGF.

We next investigated whether VEGF and EGFR are also required for tumor progression. After wounding-induced tumors had developed, mice were treated with tamoxifen to delete VEGF and/or EGFR as outlined in Figure 3B (therapeutic trial). While VEGF expressing tumors continued growing, deletion of VEGF in EGFR$^{wa2/wa2}$ K5-SOS tumors impaired further tumor growth and tumor sizes remained essentially unchanged throughout the entire treatment period (Fig. 3B). Similar results were obtained if the EGFR was deleted at the same time as VEGF (Fig. 3B). These results show that VEGF and EGFR are required both in the initial phase of tumor formation as well as during tumor progression.

Molecular analysis of tumor cells lacking EGFR and VEGF

We next investigated the cellular and molecular mechanism underlying the lack of tumor development in K5-SOS transgenic mice in the absence of EGFR and VEGF. In particular, we examined whether the observed phenotypes were indirectly mediated by VEGF affecting tumor angiogenesis or if VEGF would have an autocrine effect directly on tumor cells. To discriminate between these possibilities, primary keratinocytes isolated from mice of various genotypes were analysed in culture. Western blot analysis revealed that the levels of Erk1/2 phosphorylation were always high in the presence of the K5-SOS transgene and were not affected by the presence of a hypomorphic EGFR and/or by VEGF deletion (Fig. 4A). Erk phosphorylation was also increased in K5-SOS expressing tumors irrespective of the status of EGFR and VEGF (Fig. 4E). In contrast, the phosphorylation of other MAP-kinases such as p38 and JNK was comparable among all genotypes and not affected by K5-SOS expression (Fig. 4A). The phosphorylation of Akt was impaired in $EGFR^{wa2/wa2}$ K5-SOS transgenic keratinocytes but not further affected by the additional deletion of VEGF (Sibilia et al., 2000) and data not shown). These results demonstrate that SOS expression leads to increased Erk1/2 activation both *in vivo* and *in vitro*.

In accordance with the *in vivo* results, BrdU labeling of primary keratinocytes revealed that K5-SOS expression significantly increased the proliferation of keratinocytes, which was prevented by the additional deletion of VEGF (Fig. 4B). Interestingly, VEGF deletion dramatically reduced the proliferation of EGFR wild-type as well as $EGFR^{wa2/wa2}$ keratinocytes even in the absence of K5-SOS whereas reduced EGFR signaling alone did not affect keratinocyte proliferation (Fig. 4B). If both EGFR and VEGF were deleted proliferation was even more reduced (Fig. 4B). These results demonstrate that VEGF deletion reduces the proliferation of keratinocytes and K5-SOS-transformed epidermal tumor cells in a cell-autonomous manner. The number of apoptotic cells was similar in keratinocytes of all genotypes and RNAse protection assays revealed that mRNA levels of various caspases were not affected in epidermal

cells neither by deletion of EGFR nor VEGF (data not shown). Several anti-apoptotic genes were highly upregulated in keratinocyte cultures when compared to epidermis (Suppl. Fig. 3A). Moreover, IL18 which is known to have an anti-apoptotic effect on keratinocytes, was highly induced in culture by the K5-SOS transgene (Suppl. Fig. 3B). These results might explain why EGFR mutant keratinocytes *in vitro* do not display increased apoptosis as EGFR mutant epidermal cells *in vivo* (Sibilia et al., 2000).

From our results it seems that VEGF can stimulate keratinocyte proliferation. We therefore investigated whether VEGF receptors are expressed in primary keratinocytes and epidermal tumors. Interestingly, Flt1 and its coreceptor Nrp1 were expressed on keratinocytes, whereas Flk1 could not be detected (Fig. 4C,D and data not shown). While Flt1 expression was not affected by reduced EGFR signaling, Nrp1 levels were significantly lower in keratinocytes from EGFR mutant mice (Fig. 4C,D). Interestingly, the expression of both Nrp1 and Flt1 was highly upregulated by the K5-SOS transgene (Fig. 4C,D). However, K5-SOS-dependent Nrp1 induction was slightly reduced in EGFR mutant epidermal cells suggesting that Nrp1 expression is under the control of EGFR signaling (Fig. 4C,D). Importantly, also K5-SOS tumors from EGFR mutant mice expressed lower levels of Nrp1 protein, whereas Flt1 levels were high in all K5-SOS tumors (Fig. 4F,G) further showing that epidermal cells express Nrp1 and Flt1 which are highly upregulated in K5-SOS-transformed cells. Taken together, these results demonstrate that K5-SOS expression leads to upregulation of Flt1, Nrp1 and VEGF expression possibly via the Erk pathway resulting in increased VEGF-dependent epidermal cell proliferation.

Autocrine VEGF-Flt1 stimulation controls tumor cell proliferation

Next we examined if VEGF mediates its activity by activating Flt1. Interestingly, the levels of Flt1 phosphorylation were clearly higher in keratinocytes expressing K5-SOS, which showed high Flt1 and Nrp1 expression, than in wild-type cells (Fig. 5A). Moreover, compared to wild-type cells, low levels of Flt1 phosphorylation could be

detected already in starved K5-SOS keratinocytes, suggesting that VEGF produced by K5-SOS cells might be responsible for this effect (Fig. 5A). Furthermore, VEGF stimulation induced Flt1 phosphorylation in K5-SOS expressing cells, but not in wild-type cells (Fig. 5A). Interestingly, also stimulation with EGF led to tyrosine phosphorylation of Flt1 already after 10 minutes and persisted for 4 hours suggesting an autocrine mechanism mediated by VEGF (Fig. 5A). In K5-SOS expressing epidermal cells stimulation with VEGF also significantly induced Akt and Erk1/2 phosphorylation as well as cell proliferation (Fig. 5B,C). In contrast, K5-SOS negative keratinocytes expressing low levels of VEGFRs were only poorly stimulated by VEGF (Fig. 5C). These results show that VEGF acts via stimulation of Flt1 and that the ability of VEGF to stimulate downstream signaling pathways correlates with the levels of VEGFR expressed on epidermal cells.

To further prove that autocrine VEGF-Flt1 signaling is responsible for tumor cell proliferation, we knocked-down (KD) both Flt1 and Nrp1 in epidermal cells. The KD of either Flt1 or Nrp1 resulted in a strong reduction in the number of proliferating cells further demonstrating that VEGF affects epidermal cell proliferation via activation of these receptors (Fig. 5D,E,F). Moreover, wild-type and K5-SOS transgenic epidermal cells were also treated with the intracellular VEGFR kinase inhibitors Sunitinib (Potapova et al., 2006; Zhou et al., 2008) and BI-1120 (Hilberg et al., 2008), and with the extracellular VEGFR inhibitors αVEGFR1 (neutralizing antibody) and $Flt_{2\text{-}11}$ (blocking peptide) (Tan et al., 2001). All approaches revealed that cell proliferation was decreased along with the secretion of VEGF (except for $Flt_{2\text{-}11}$), the expression of Flt1 (except for $Flt_{2\text{-}11}$) and Nrp1 (Fig. 5G-J). This was associated with a moderate to strong inhibition of Erk activation (Suppl. Fig. 3E). The observation that the intracellular inhibitors had a stronger effect than the extracellular suppressors can be either due to the broader spectrum of inhibition by Sunitinib and BI-1120 or to intracellular VEGFR activation which is not inhibited by the extracellular suppressors. Together our data

suggest that autocrine epidermal cell proliferation via Flt1 can occur intracellularly as well as via secreted VEGF.

K5-SOS expressing keratinocytes proliferate more and show increased Erk1/2 activation as well as increased expression of VEGF and its receptors Flt1 and Nrp1. To investigate if the Erk1/2 pathway might control cell proliferation via the expression of VEGF and its receptors, keratinocytes were treated with the MEK inhibitors CI-1040/PD-184352 (Bain et al., 2007; Liu et al., 2007; Lorusso et al., 2005; Lunghi et al., 2008) and UO126 (Bain et al., 2007) which inhibited Erk1/2 phosphorylation (Suppl. Fig. 3C,D). Inhibition of Erk1/2 significantly affected keratinocyte proliferation and VEGF secretion even when K5-SOS was expressed (Fig. 5G,H). Similarly, the levels of Flt1 and Nrp1 were also significantly decreased after treatment with UO126 or CI-1040 (Fig. 5I,J). Taken together these results demonstrate that K5-SOS expression leads to constitutive activation of Erk1/2 resulting in increased expression of VEGF and its receptors which in turn lead to increased tumor cell proliferation by strengthening the Erk pathway.

Combined anti-VEGFR and anti-EGFR therapy impairs K5-SOS- and RasV12-dependent tumorigenesis

Anti-EGFR and anti-angiogenic therapies are being employed for the treatment of several human cancers (Ciardiello and Tortora, 2008; Ferrara, 2005). We next investigated whether pharmacological inhibition of EGFR and VEGF signaling has a synergistic effect on inhibiting tumor growth. A novel irreversible EGFR inhibitor (BI-2992; Boehringer Ingelheim) (Eskens et al., 2008; Li et al., 2008; Riely, 2008) and a reversible VEGFR inhibitor (BI-1120; Boehringer Ingelheim)(Hilberg et al., 2008), which are currently in phase III clinical trials were tested in this study. K5-SOS transgenic mice which had already developed skin tumors were treated either with one or in combination with both classes of inhibitors. Whereas treatment with the VEGFR inhibitor only mildly inhibited tumor growth, the EGFR inhibitor significantly impaired

tumor growth and the tumor size remained unchanged throughout the entire treatment period (Fig. 6A). These findings were very similar to what observed with the respective genetic deletions (Fig. 3B and data not shown). Interestingly, combined therapy with the EGFR and VEGFR inhibitors BI-1120 and BI-2992 showed a synergistic inhibitory effect on tumor growth and some of the tumors showed complete regression (Fig. 6A). Importantly, when single EGFR inhibitor treatment was stopped tumors started to grow again and their volumes increased very fast. However, tumor re-growth was delayed in tumors treated with both EGFR and VEGFR inhibitors likely because neovascularization of the tumor tissue has to occur. These results show that pharmacological inhibition of the EGFR pathway effectively reduces tumor growth and that the combination therapy with EGFR and VEGFR inhibitors is more effective leading to almost complete tumor regression.

Similarly to what was observed in the genetic experiments, immunohistochemical analysis of papilloma sections showed that tumors treated with the EGFR inhibitor were more differentiated whereas the VEGFR inhibitor did not affect tumor cell differentiation (Suppl. Fig. 2B and data not shown). Moreover, administration of EGFR or VEGFR inhibitors resulted in a significant reduction of blood vessel density (Fig. 6C,D). This effect was more pronounced in papillomas treated in combination with both BI-1120 and BI-2992. Interestingly, therapy with both inhibitors dramatically decreased the number of proliferating tumor cells (Fig. 6C,E). Furthermore, pharmacological inhibition of EGFR signaling led to an increase in apoptosis and this effect was even more pronounced when tumors were additionally treated with the VEGFR inhibitor (Fig. 6C). These results demonstrate that pharmacological inhibition of EGFR and VEGFR signaling has a similar effect on tumor growth as genetic deletion of EGFR and VEGF (Sibilia et al., 2000) and data not shown). Both approaches led to a significant reduction in tumor cell proliferation suggesting that VEGF signaling cell-autonomously controls the proliferation of epidermal tumor cells. Furthermore, the increase in

apoptosis in tumors treated with EGFR inhibitors confirms that EGFR acts as a survival factor for tumor cells.

Many tumors harbor activating mutations of the Ras oncogene. To investigate whether besides K5-SOS-dependent tumors, EGFR and VEGF inhibitors can also be employed to treat Ras-dependent tumors, we induced subcutaneous tumors in nude mice with RasV12- transformed NIH3T3 cells (Sibilia et al., 2000). After 10 days when 100% of the mice had developed tumors, mice were treated either with the VEGFR inhibitor BI-1120 or the EGFR inhibitor BI-2992 alone or in combination. Similar to the results obtained in the K5-SOS tumor model, both inhibitors significantly impaired tumor development when compared to vehicle treatment (Fig. 6B). Also in this model, anti-EGFR therapy was more efficient than the treatment with BI-1120 alone and the best tumor-inhibitory effect was observed when both compounds were used in combination (Fig. 6B). These results demonstrate that anti-EGFR or anti-angiogenic therapies alone or in combination can be efficiently employed to treat tumors harboring mutations in the Ras pathway.

Human epidermal tumors and cell lines express Flt1 and its coreceptor Nrp1

To determine the relevance of these findings to human epidermal cancer, we examined the expression of Flt1 and Nrp1 in human epidermal tumors. Sections from 194 basal cell carcinoma (BCC) and 163 squamous cell carcinoma (SCC) patients as well as 95 normal skin samples were analysed by immunohistochemistry (Fig. 7A-E). In normal human skin, weak intracellular Flt1 staining was observed in 40% of the samples whereas the remaining 60% did not show any Flt1 expression (Fig. 7A,E). In BCC samples the pattern of Flt1 expression was similar to normal skin: 40% of the samples showed weak intracellular Flt1 staining, while in the remaining samples Flt1 expression was negligible (Fig. 7E). In contrast, 80% of SCC samples were positive for Flt1, whereas 20% did not express Flt1. Among the Flt1 positive SCC samples, 21% displayed strong cytoplasmic staining and weak intracellular expression was found in

60% of patient material (Fig. 7D,E). Overall, strong Flt1 staining at the cell membrane was observed in 18% of SCC samples whereas Flt1 membrane staining was observed only in 0.5% of BCC patients and normal skin was always negative (Fig. 7C,E). Moreover, we also isolated RNA from human SCC biopsies and qRT-PCR analysis revealed that Flt1 expression was increased in skin cancer biopsies compared to normal skin (Suppl. Fig 4A). Nrp1 expression was detectable in human normal skin, BCC and SCC but its expression levels did not correlate with the grade of malignancy (data not shown). These results show that Flt1 is upregulated in the majority of human SCC but not in BCC.

To address the functional relevance of Flt1 upregulation in human SCC we analyzed Flt1 expression in 6 human SCC cell lines and found that 2 of them expressed low levels and 4 high levels of Flt1 (Suppl. Fig. 4B). Flt1 protein was also detectable in the 4 high expressor lines (Suppl. Fig. 4C and data not shown) and VEGF stimulation of lines SCCO11 and SCC13 with the highest level of Flt1 expression led to receptor phosphorylation (Suppl. Fig. 4C). SCCO11 and SCC13 were treated with the intracellular VEGFR kinase inhibitors Sunitinib and BI-1120, and with the extracellular VEGFR inhibitors αVEGFR1 and $Flt_{2\text{-}11}$. Similarly to what observed with mouse epidermal cells, all the compounds significantly reduced cell proliferation in both SCC cell lines except for αVEGFR1 in SCCO11 (Fig. 7F). Flt1 expression and activation was also inhibited by both the intracellular VEGFR kinase inhibitors but not by the extracellular suppressors (Fig. 7G, Suppl. Fig. 4C). In contrast, Nrp1 expression was not affected (Fig. 7H), which correlates with the results in human SCC biopsies, where Nrp1 was expressed but its levels did not correlate with the grade of malignancy. Also in human SCC, the effects of the intracellular kinase inhibitors were stronger than the extracellular suppressors suggesting that autocrine intracellular VEGFR signaling might contribute to the observed phenotypes. A moderate reduction in Erk activation was also detectable after treatment with VEGFR inhibitors (Suppl. Fig. 4D). In addition, similarly to what was observed with mouse tumor cells, treatment of the SCC cell lines with the

MEK inhibitors U0126 or CI-1040 resulted in significant reduction of proliferation, Flt1 and Nrp1 expression (Fig. 7F-H, Suppl. Fig 4E,F) suggesting that also in human cells VEGFR expression and cell proliferation is controlled via the Erk pathway. These results demonstrate that autocrine VEGF-Flt1 signaling controls the proliferation of human SCC cells and might therefore be an attractive target for therapeutic intervention in SCC patients.

DISCUSSION

It is accepted that VEGF is produced by tumor cells and acts on neighboring VEGFR expressing endothelial cells to promote neovascularization for continued tumor growth and metastatic spread (Bergers and Benjamin, 2003; Ferrara et al., 2003; Ferrara and Kerbel, 2005). Our results show a new function for VEGF besides its classical role as an angiogenic factor. We demonstrate that *in vivo* in a K5-SOS-dependent mouse skin tumor model autocrine VEGF is required for epithelial tumor cell proliferation in a cell-autonomous manner. Indeed, K5-SOS expression leads to Erk-dependent upregulation of VEGF and its receptors Flt1 and Nrp1 in tumor cells. Therefore, epidermis-specific VEGF deletion results in reduction of K5-SOS-dependent tumor burden not only by regressing tumor vessels but also by affecting tumor cell proliferation. Surprisingly, tumor development was completely inhibited in the absence of epidermal VEGF and EGFR expression demonstrating a synergistic, tumor-promoting effect of EGFR and VEGF signaling in neoplastic cells.

It had previously been shown that expression of dominant negative EGFR impaired Ha-ras-dependent tumor growth by affecting tumor vessels which was likely due to reduced VEGF expression (Casanova et al., 2002). However, we did not observe differences in blood vessel density in K5-SOS transgenic tumors of wild-type and mutant EGFR background and VEGF expression was similar in both genotypes. It seems that the expression of an activated form of SOS can overcome the need for EGFR for efficient VEGF production. Only in the complete absence of VEGF expression in the epidermis, the number of blood vessels present in K5-SOS skin tumors was significantly reduced. Moreover, inducible deletion of VEGF in the epidermis at different stages of tumor development revealed that VEGF signaling is limiting tumor growth both during tumor initiation and progression.

Interestingly, Nrp1 expression was reduced in EGFR mutant cells, even in the presence of the K5-SOS transgene, suggesting that Nrp1 expression is controlled by EGFR signaling. Indeed, several studies have shown that EGFR signaling modulates

the expression of Nrp1 by inhibiting the expression of the tumor repressor Neuron Restrictive Silencer Factor (NRSF), a transcriptional repressor of Nrp1 (Akagi et al., 2003; Kurschat et al., 2006). However, NRSF expression was not affected in K5-SOS tumors suggesting a different Nrp1-regulating mechanism (data not shown). AP-1, Sp1 and CCAAT elements are also present in the Nrp1 promoter (Rossignol et al., 2003). We exclude that EGFR signaling modulates Nrp1 expression via AP-1 transcription factors, since their expression was not affected in EGFR mutant cells (data not shown). Independent studies have shown that the expression of Nrp1 is also regulated by VEGF signaling (Oh et al., 2002). Therefore, EGFR signaling might affect Nrp1 expression also indirectly by inducing VEGF production.

We demonstrate that pharmacological inhibition of VEGFR in tumor bearing K5-SOS transgenic mice delays tumor growth whereas treatment with EGFR inhibitors significantly reduced tumor growth. Importantly, treatment with VEGFR and EGFR inhibitors together was more efficient and, in some cases, resulted in almost complete tumor regression demonstrating that combined therapies are more effective for curing cancer. In accordance with the results obtained with genetic VEGF deletion, pharmacological inhibition of VEGFR resulted in a striking reduction of proliferating cells within the tumors. The increase in apoptosis within the tumor tissue upon administration of EGFR inhibitors confirmed once more that EGFR provides a survival signal to epithelial tumor cells. Interestingly, inhibitor treatment had a similar inhibitory effect on RasV12-mediated tumors demonstrating that at least in the early stages of tumor development such therapies could also be effective to treat tumors with activating mutations in the Ras pathway.

Both genetic as well as pharmacological inhibition of the VEGFR pathway in K5-SOS mice resulted in strongly impaired tumor cell proliferation. This could be a secondary effect resulting from reduced angiogenesis caused by impaired VEGF signaling which would prevent sufficient tumor nourishment. However, we observed that proliferation upon genetic VEGF deletion was also reduced *in vitro* suggesting that VEGF has an

additional cell-autonomous effect on epidermal cells. Indeed, we could demonstrate that wild-type as well as K5-SOS transgenic primary epidermal cells express the VEGF receptors Flt1 and Nrp1. Interestingly, K5-SOS highly upregulated the expression of these two receptors in an Erk-dependent manner. Indeed, this increase in expression of VEGF and its receptors could be blocked by inhibiting ERK1/2 phosphorylation, which was always elevated in the presence of K5-SOS. Moreover, pharmacological or siRNA-mediated inhibition of Flt1 and Nrp1 in epidermal cells greatly impaired their proliferation potential further demonstrating that autocrine VEGF-Flt1 signaling controls tumor cell proliferation independently of angiogenesis. Initially, VEGF receptors were thought to be expressed only on endothelial cells. However, recent studies have suggested that these receptors may also be expressed on tumor cells and epithelial cells (Bachelder et al., 2001; Chung et al., 2006; Fan et al., 2005; Lacal et al., 2000; Lee et al., 2007b; Mercurio et al., 2004; Parikh et al., 2004). Flt1 was detected in NHEK cells and in the epidermis of BALB/c mice (Wilgus et al., 2005). Nrp1 expression was found in human suprabasal epidermis (Kurschat et al., 2006). Furthermore, human melanocytes express Nrp1, Flt1 and Flk1 upon stimulation with the phorbol ester TPA and Flk1 was phosphorylated in these cells upon VEGF addition (Kim et al., 2005).

Interestingly, previous studies have shown that an autocrine signaling loop for VEGF, which is mostly triggered inside the cell, is required for the survival of haematopoietic stem cells and endothelial cells (Gerber et al., 2002; Lee et al., 2007a). We could show that K5-SOS transgenic epidermal cells, which highly express VEGF and its receptors Flt1 and Nrp1, had increased levels of phosphorylated Flt1 compared to wild-type cells. Moreover, stimulation of K5-SOS epidermal cells with VEGF resulted in tyrosine phosphorylation of Flt1 as well as activation of Erk and increased proliferation. Interestingly, also stimulation with EGF led to Flt1 tyrosine phosphorylation in K5-SOS expressing cells suggesting an autocrine mechanism mediated by VEGF. In contrast, stimulation of wild-type epidermal cells with VEGF or EGF did not significantly induce any of these effects. Interestingly, in wild-type cells a significant reduction in cell

proliferation was only observed with intracellular VEGFR inhibitors suggesting that signaling may occur intracellularly. Also in K5-SOS cells the intracellular inhibitors had a stronger effect than the extracellular suppressors. This can be either due to the broader spectrum of inhibition by Sunitinib and BI-1120 or to intracellular VEGFR activation which is not inhibited by the extracellular suppressors. Together our data suggest that autocrine Flt1 activation occurs intracellularly as well as via VEGF secretion leading to epidermal cell proliferation.

Several studies have reported Nrp1 expression on a variety of cancer cells and correlated its expression with poor prognosis (Bielenberg et al., 2006; Hansel et al., 2004; Parikh et al., 2004). However, there were also studies showing the opposite illustrating the complex function of Nrp1 in tumors (Gray et al., 2005; Kamiya et al., 2006). We found that Nrp1 was expressed in human BCC and SCC, but its expression did not correlate with the grade of malignancy. In BCC samples the pattern of Flt1 expression was also similar to normal skin. However, we observed that 80% of SCC samples were positive for Flt1 and among those 18% had strong Flt1 staining at the cell membrane, while 21% showed highly increased cytoplasmic Flt1 expression. In accordance with our finding, Lee et al. reported that Flt1 is predominantly expressed intracellularly in breast cancer cell lines and primary breast cancer tumors (Lee et al., 2007b). Moreover, they provided evidence that VEGF is an internal autocrine survival factor for breast cancer cells by binding to Flt1. Importantly, we could show that various human SCC cell lines express increased levels of Flt1 which is tyrosine phosphorylated upon VEGF stimulation. Moreover, treatment with VEGFR inhibitors resulted in a significant reduction of proliferation whereby intracellular inhibitors were more effective than extracellular suppressors. Therefore, our data demonstrate that, similarly to what observed in mouse tumor cells, also in human SCC autocrine cell proliferation controlled by Flt1 can occur intracellularly as well as by VEGF secretion. Thus, VEGFR1 inhibitors might be an attractive target for therapeutic intervention of different types of cancers.

In conclusion we demonstrate that constitutive active SOS activates the ERK pathway leading to high expression of VEGF and its receptors Flt1 and Nrp1. A similar mechanism might be utilized by human epithelial tumors harboring oncogenic Ras signaling. Increased VEGF-Flt1/Nrp1 induces tumor angiogenesis and potentiates cell-autonomous tumor cell proliferation (Fig.7I). Therefore VEGF/VEGFR inhibition not only impairs angiogenesis but also tumor cell proliferation. Moreover, in K5-SOS mice lacking both epidermal EGFR and VEGF, cells undergo apoptosis due to reduced EGFR signaling and therefore papilloma formation is completely blocked (Fig.7I). This study represents the first demonstration of a synergistic action of EGFR and VEGF signaling in tumor cells and provides a molecular explanation why combined anti-EGFR and anti-VEGFR therapies might be more efficient than single treatments.

EXPERIMENTAL PROCEDURES

Mice

VEGF$^{f/f}$ (Gerber et al., 1999), K5-SOS (Sibilia et al., 2000), EGFR$^{wa2/wa2}$ (Sibilia et al., 2000), EGFR$^{f/f}$ (Natarajan et al., 2007), K5-Cre (Tarutani et al., 1997) and K5-CreER^{T} (Indra et al., 1999) mice were previously described. For inducible VEGF and/or EGFR deletion adult mice were injected intraperitoneally with 1mg of tamoxifen (Sigma; sunflower seed oil/ethanol mixture (10:1) at 10mg/ml) per day according to the schemes indicated in the figures. Mice were kept in the animal facility of the Medical University of Vienna in accordance with institutional policies and federal guidelines.

Pharmacological inhibition of tumor growth

Inhibitors used in this study were kindly provided by Boehringer Ingelheim Austria. Inhibitors were applied orally to K5-SOS transgenic mice at the following concentrations: BI-1120 100mg/kg/day; BI-2992 20mg/kg/day; for combined therapy 75mg/kg/day BI-1120 and 15mg/kg/day BI-2992. The carrier Natrosol was given to untreated control mice. To induce RasV12-dependent tumor formation, athymic nu/nu mice were subcutaneously inoculated in the flank area with 10^6 RasV12-transformed NIH3T3 cells (Sibilia et al., 2000). After tumors had developed, mice were treated with inhibitors as described above.

Human SCC and mouse epidermal cell cultures, BrdU staining and inhibitor treatment

Mouse epidermal cells were isolated as previously described and cultured on vitrogen-fibronectin coated dishes in low calcium MEM medium (Sigma) containing 8% chelated FCS (Sibilia et al., 2000). The human SCC cell lines SCC4, SCC9 (ATCC) were kindly provided by Erwin Tschachler and the SCCO11, SCCO12, SCC13 and SCCO22 lines by Gian-Paolo Dotto and cultured as previously described (Lefort et al., 2007). Mouse or human cultures at 80% confluency were left untreated or stimulated with 20ng/mL

EGF (Roche) or 30ng/mL VEGF (R&DSystems) for 5 minutes and harvested for RNA or protein analysis. For VEGFR or ERK inhibition, 80% confluent cells were treated with the VEGFR inhibitors Sunitinib (1µM; Pfizer), BI-1120 (500nM; Boehringer Ingelheim), anti-VEGFR1 antibody AF471 (2,5µg/mL; R&D Systems), VEGF blocking-peptide Flt_{2-11} (1µg/mL; Calbiochem), and the Erk inhibitors CI-1040 (500nM; Pfizer), U0126 (10µM; Promega) or DMSO (Fluka) alone for 12-48 hours before supernatants, protein or RNA lysates were harvested for further analyses. For proliferation analysis of mouse keratinocytes 80% confluent cells were incubated with the respective inhibitors or VEGF (100ng/mL; R&DSystems) for 48 hours before pulsing with 10µM BrdU (Roche) for 4 hours, fixation with 70% ethanol and immunofluorescent staining with an anti-BrdU antibody according to the manufacturer's instructions (Becton Dickinson). $BrdU^+$ cells from 6-10 randomly chosen fields of at least 3 independent samples were counted. For proliferation analysis in human SCC cell lines, 80% confluent cells were incubated with the respective inhibitors for 24 hours before pulsing with 20µM BrdU (BD Pharmingen) for 6 hours. Cells were trypsinized and stained with the APC BrdU Flow Kit (BD Pharmingen) and the number of proliferating cells was analyzed on a LSR-II Flow cytometer (BD Biosciences).

Knock-down of Flt1 and Nrp1 in epidermal cells

4 different miRNA oligos corresponding to Flt1 or Nrp1 and the non-target negative controls were purchased from Invitrogen and cloned into the pcDNA^{TM}6.2-GW/EmGFP-miR vector (Invitrogen) containing an EGFP marker with the BLOCK-iT^{TM} Pol II miR RNAi Expression Vector Kit according to the manufacturer's instructions. The vectors were transfected into primary epidermal cells with Fugene HD (Roche). 24h after transfection keratinocytes were pulsed with 20µM BrdU for 6h. Transfection efficiencies were measured by the expression of EmGFP by flow cytometric analysis and ranged from 40 to 50%. To quantify proliferation cells were trypsinized and stained with the APC BrdU Flow Kit (BD Pharmingen). The percentage of proliferating transfected

(EGFP$^+$/BrdU$^+$) cells was analyzed with a LSR-II flow cytometer (BD Biosciences) using FACSDiva software (BD Biosciences). Proliferation rates were compared to the number of proliferating cells transfected with the negative control vector. Expression of Flt1 or Nrp1 upon miRNA knock-down was quantified by qRT-PCR.

Isolation of skin endothelial cells

Shaved dorsal and ventral skin was minced into pieces and incubated in collagenase (Wortington) for 60 min at 37°C. The resulting cell suspension was filtered and stained with anibodies against CD31, CD144 (BD Biosciences) and CD45 (Coulter). Cellular suspensions were washed and CD31$^+$ CD144$^+$ CD45$^-$ endothelial cells (ECs), CD31$^-$ CD144$^-$ CD45$^+$ leukocytes and CD31$^-$ CD144$^-$ CD45$^-$ stromal cells and keratinocytes (E.K., unpublished observations) were sorted to a purity of > 95% on a FACSAria Cell Sorter (BD Biosciences). Sorted cells were lysed in TRI Reagent (Sigma) containing 1% β-mercaptoethanol (MERCK) and RNA was isolated according to standard procedures.

Histological analysis

Mouse tissues were embedded in OCT (Sakura), 5 µm cryosections were cut and fixed in acetone or 1% PFA before processing. Epidermal ear sheets were prepared by separating epidermis from dermis with 3.5% ammoniumthiocyanate and fixed in acetone. For immunohistochemistry and immunofluorescent stainings the following antibodies were used: anti-mouse CD31/PECAM-1 (BD PharMingen), anti-Ki67 (Novocastra), anti-EGFR (Santa Cruz), anti-keratin 1 and anti-keratin 14 (Babco), anti-phospho-p44/42 (New England Biolabs), anti-Flt1 and anti-Nrp1 (Santa Cruz) and secondary antibodies purchased from Molecular Probes and Vector Laboratories. In order to investigate the average blood vessel density, a computer-assisted morphometric analysis was performed with the MetaMorph Imaging System. For antigen retrieval, paraffin embedded human tissue arrays were treated with Target

Retrieval Solution (Dako) and processed further according to the manufacturer's recommendation. Isotype IgG from rabbit serum (Sigma) and biotinylated anti-rabbit IgG were used as controls (Vector Laboratories).

Total RNA isolation, RT-PCR analysis and RNase Protection Assay (RPA)

Total RNA from epidermis, cultured epidermal cells, SCC cell lines or biopsies were isolated with TRIzol Reagent (Invitrogen). RPAs were performed with the Multi-Probe RNase Protection Assay System (BD Biosciences) according to the manufacturer's instructions. cDNA synthesis was performed with SuperScript First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. Primers used for semiquantitative RT-PCR analysis: *gapdh* 5'-CTCATGACCACAGTCCATGC-3' and 5'-CACATTGGGGGTAGGAACAC-3', *vegf* 5'-GCCCTGGAGTGCGTGCCCACGTCAGAGAGCA-3' and 5'-TGGCGATTTAGCAGCAGATA-3'. qRT-PCR was performed using the LightCycler FastStart DNA Master^{PLUS} SYBR Green I kit together with the LightCycler 2.0 System (Roche) with the following primers for murine genes: *flt1* 5'-GAGGAGGATGAGGGTGTCTATAGGT-3' and 5'-GTGATCAGCTCCAGGTTTGACTT-3'; *nrp1* 5'-ACAAGGAGTGGATCCAGGTG-3' and 5'-ACATCTGTGGGGTTGGTGTT-3'. *pbgd (internal standard)* 5'-GCACTTTTCTCTGGCAAGGT-3' and 5'-GTCTCCTGCAGGCTCTATCG-3'; and for human genes: *huvegf* 5'-ATCTTCAAGCCGTCCTGTGT-3' and 5'-GCATTCACATCTGCTGTGCT-3'; *huflt1* 5'-ATCATTCCGAAGCAAGGTGTG-3' and 5'-AAACCCATTTGGCACATCTGT-3'; *hunrp1* 5'-CCACAGTGGAACAGGTGATG-3' and 5'-GCACGTGATTGTCATGTTCC-3'; *hugapdh (internal standard)* 5'-GGAAGGTGAAGGTCGGAGTCA-3' and 5'-GTCATTGATGGCAACAATATCCACT-3'.

Southern blot analysis

Southern blot analysis was performed according to standard protocols. Genomic DNA isolated from keratinocytes was digested with AccI. Floxed and delta alleles of *vegf* were detected with a probe kindly provided by J. Haigh.

Western blot analysis and immunoprecipitation

Cells were starved for 24h in 0.5% FCS or serum-free medium prior to growth factor stimulation. Protein lysates were prepared as previously described (Sibilia et al., 2000). For immunoprecipitation, 1.5mg protein lysates were incubated with anti-Flt1 antibodies pre-coupled to Ultralink immobilised Sepharose beads (Pierce) according to the manufacturer's recommendations and incubated overnight at 4°C. The bead-antibody-protein complexes were collected by centrifugation, washed 3x in lysis buffer and resuspended in denaturing protein loading buffer. Proteins were separated by SDS-PAGE and transferred to PVDF membranes (Millipore). Western blot analysis was performed as previously described (Sibilia et al., 2000) with antibodies detecting phospho-JNK, JNK, phospho-p38, p38 (Cell Signaling), phospho-p44/42 (New England Biolabs), ERK1/ERK2 (Santa Cruz), phospho-tyrosine (Cell Signaling), Flt1 (Abcam), actin and tubulin (Sigma). Membranes were reprobed after stripping in 62.5mM Tris-HCl (pH 6.8), 2% SDS, 100mM β-mercaptoethanol at 55°C for 30min.

ELISA

Mouse VEGF Immunoassay (Quantikine, R&DSystems) was performed according to the manufacturer's instructions with 48 hour-old supernatants collected from 80% confluent keratinocyte cultures or with 40µg protein of epidermal cell lysates.

Statistical methods

All experiments were repeated at least twice and done in triplicates. Data were evaluated using a Student's two-tailed t test. $p < 0.05$ was taken to be statistically significant. In Figures 1C and 1E data were analyzed by a Log-rank (Mantel-Cox) test.

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FIGURE LEGENDS

Figure 1. Effect of mutant EGFR and VEGF deletion on K5-SOS-dependent skin tumor formation

(A) RNase protection assay showing mRNA expression levels of VEGF and GAPDH in keratinocytes isolated from mice of the indicated genotypes. **(B)** Representative ELISA showing secreted VEGF in supernatants of cultured keratinocytes of the indicated genotypes. No VEGF is detected in keratinocytes isolated from $VEGF^{flf}$ K5-Cre ($VEGF^{\Delta ep}$) mice. **(C,D)** Tumor incidence (C) and volume measured at 2 months (D) in $EGFR^{wa2/+}$ K5-SOS mice in the presence or absence of VEGF. **(E,F)** Tumor incidence (E) and volume at 12 months of age (F) in $EGFR^{wa2/wa2}$ K5-SOS mice lacking one or both VEGF alleles in the epidermis. Only skin lesions ≥0.02 cm^3 were scored as tumors in all groups. **(G)** Kinetic of tumor growth measured for 35 days after wounding the tip of the ear of $EGFR^{wa2/wa2}$ K5-SOS (black circle) and $EGFR^{wa2/wa2}$ $VEGF^{f/+}$ K5-Cre K5-SOS mice (grey triangle). No tumors developed in $EGFR^{wa2/wa2}$ $VEGF^{\Delta ep}$ K5-SOS mice (white rectangle). **(H-J)** Macroscopic appearance of ears 35 days after wounding of $EGFR^{wa2/wa2}$ K5-SOS (H), $EGFR^{wa2/wa2}$ $VEGF^{f/+}$ K5-Cre K5-SOS (I) and $EGFR^{wa2/wa2}$ $VEGF^{\Delta ep}$ K5-SOS (J) mice. Data represent mean ± SEM. * p≤0.05; ** p≤0.005; *** p≤0.0005.

Figure 2. Blood vessel density and epidermal cell proliferation in K5-SOS-dependent papillomas lacking VEGF

(A) Immunofluorescent staining with antibodies against CD31 and Ki67 of spontaneous papillomas from $EGFR^{wa2/+}$ K5-SOS and $EGFR^{wa2/+}$ K5-SOS $VEGF^{\Delta ep}$ mice and of wounding-induced tumors from $EGFR^{wa2/wa2}$ K5-SOS and $EGFR^{wa2/wa2}$ $VEGF^{f/+}$ K5-Cre K5-SOS mice. **(B)** Computer-assisted morphometric analysis showing the average vessel area on αCD31-stained tumor sections isolated from mice indicated in A. Data represent the average vessel area ± SEM. **(C)** Quantification of Ki67 positive cells on

sections from three independent tumors of mice indicated in A. Data represent mean ± SEM. * p≤0.05; *** p≤0.0005.

Figure 3. Inducible deletion of EGFR and VEGF in K5-SOS transgenic mice

(A) Kinetic of tumor growth measured in mice of the indicated genotypes for 38 days after wounding the tip of the ear. All mice had been treated with tamoxifen 12 times before wounding and afterwards twice a week for maintenance as indicated in the scheme (prevention trial). **(B)** Kinetic of tumor growth of established wounding-induced tumors of the indicated genotypes measured for two weeks. After tumors had developed, all mice were treated daily with tamoxifen as indicated in the scheme (therapeutic trial). **(C)** Immunofluorescent stainings for EGFR on tumor sections showing that EGFR was efficiently deleted in $EGFR^{\Delta epER}$ mice after tamoxifen treatment. **(D)** ELISA showing VEGF present in protein lysates of keratinocytes isolated from mice used in A and B. Data represent mean ± SEM. * p≤0.05; ** p≤0.005.

Figure 4. VEGFR expression and proliferation in epidermal cells and tumors

(A) Western blot analysis showing phospho-Erk1/2, phospho-p38 and phospho-JNK levels in protein lysates from keratinocyte cultures isolated from mice of the indicated genotypes. **(B)** Quantification of proliferating, BrdU positive cells in keratinocyte cultures of the indicated genotypes. **(C,D)** qRT-PCR measuring the expression of VEGFR1/Flt1 (C) and Nrp1 mRNA (D) in cultured keratinocytes of the indicated genotypes. **(E-G)** Immunohistochemical analysis of phospho-ERK1/2 (E), Nrp1 (F) and Flt1 (G) on sections of spontaneous papillomas from $EGFR^{wa2/+}$ K5-SOS and $EGFR^{wa2/+}$ K5-SOS $VEGF^{\Delta ep}$ mice and of wounding-induced tumors from $EGFR^{wa2/wa2}$ K5-SOS and $EGFR^{wa2/wa2}$ $VEGF^{f/+}$ K5-Cre K5-SOS mice. Note that Nrp1 expression is reduced in basal tumor cells of EGFR mutant mice (B, black arrows). The data in B, C and D represent mean ± SEM. * p≤0.05; ** p≤0.005; *** p≤0.0005.

Figure 5. Inhibition of VEGFR and Erk affects proliferation and expression of VEGF and its receptors

(A) Cultured keratinocytes were either left untreated (bulk) or starved for 24 hours prior stimulation with EGF or VEGF. Protein lysates were subjected to immunoprecipitation (IP) with anti-Flt1 antibodies. Proteins were resolved by SDS-PAGE and immunoblotted (IB) with the indicated antibodies. **(B)** Quantification of proliferating, BrdU positive cells in keratinocyte cultures of the indicated genotypes upon VEGF treatment. **(C)** Western blot analysis showing ERK phosphorylation in keratinocytes stimulated with EGF and VEGF. Numbers indicate the levels of Erk1/2 activation relative to the respective controls after correction with total ERK normalized to actin (loading control). **(D**) Quantification of proliferating keratinocytes after miRNA knock-down of Flt1 and Nrp1. **(E,F)** Relative expression of Flt1 (B) and Nrp1(C) upon knock-down with two different vectors. **(G)** Quantification of proliferating keratinocytes after treatment with the VEGFR inhibitors Sunitinib, BI-1120, anti(α)-VEGFR antibody, the VEGF blocking-peptide $Flt_{2\text{-}11}$ and the Erk inhibitors CI-1040 and U0126. Shown asterisks (*) refer to comparisons with respective untreated controls; Following p values were obtained in comparisons between: $EGFR^{wa/+}$: BI-1120 with αVEGFR, p=0.01; BI-1120 with $Flt_{2\text{-}11}$, p=0.02; Sunitinib with αVEGFR, p=0,04; $EGFR^{wa2/+}$ K5-SOS: BI-1120 with αVEGFR, p=0.02; BI-1120 with $Flt_{2\text{-}11}$, p=0.05; Sunitinib with αVEGFR, p=0,02; Sunitinib with $Flt_{2\text{-}11}$, p=0.05. **(H,I,J)** Treatment with the inhibitors indicated in (G) affects VEGF protein secretion after 48h measured by ELISA (H) and Flt1 (I) and Nrp1 (J) mRNA expression measured after 12h by qRT-PCR in cultured keratinocytes. Data represent mean ± SEM. * p≤0.05; ** p≤0.005; *** p≤0.0005.

Figure 6. Combined pharmacological inhibition of EGFR and VEGFR in K5-SOS transgenic mice

(A) Relative tumor volume of K5-SOS transgenic mice treated with vehicle (Natrosol), EGFR inhibitor (BI-2992), VEGFR inhibitor (BI-1120) or combination therapy with B-

1120 and BI-2992. Arrows indicate the time of termination of the respective treatment. **(B)** Kinetic of RasV12-mediated tumor growth upon treatment with vehicle (Natrosol), EGFR inhibitor (BI-2992), VEGFR inhibitor (BI-1120) or the combination of both. **(C)** TUNEL staining and immunofluorescent staining with antibodies against CD31 and Ki67 of papillomas treated with the indicated inhibitors. **(D)** Computer-assisted morphometric analysis measuring the average vessel area on αCD31-stained tumor sections of papillomas from mice treated with the indicated inhibitors. Data represent the average vessel area ± SEM. **(E)** Quantification of Ki67 positive cells on sections from 3 independent tumors of mice treated with the indicated inhibitors. Data represent mean ± SEM. * p≤0.05; ** p≤0.005; *** p≤0.0005.

Figure 7. Flt1 is upregulated in human squamous cell carcinomas (SCC)

(A-D) Immunohistochemical stainings with antibodies against Flt1 (A,C,D) and isotype control (B) showing Flt1 expression on human normal epidermis (A) and on human SCC (C,D). **(E)** Distribution of Flt1 protein expression on human normal epidermis (epi), basal cell carcinomas (BCC) and SCC. Samples were either negative for Flt1 expression or showed a weak/strong intracellular or membrane Flt1 staining. **(F)** Quantification of proliferating SCC cells after treatment with the VEGFR inhibitors Sunitinib, BI-1120, αVEGFR antibody, the VEGF blocking-peptide $Flt_{2\text{-}11}$ and the Erk inhibitors CI-1040 and U0126. Shown asterisks (*) refer to comparisons with respective untreated controls; Following p values were obtained in comparisons between: SCCO11: BI-1120 with αVEGFR, p=0.0197; BI-1120 with $Flt_{2\text{-}11}$, p=0.0018; Sunitinib with αVEGFR, p=0,0029; SCC13: BI-1120 with αVEGFR, p=0.0037; BI-1120 with $Flt_{2\text{-}11}$, p=0.0015; Sunitinib with αVEGFR, p=0.0025; Sunitinib with $Flt_{2\text{-}11}$, p=0.0004. **(G,H)** Treatment with the inhibitors indicated in (F) affects Flt1 and Nrp1 mRNA expression (measured by qRT-PCR) in SCC cell lines after 24h. **(I)** Model of SOS/EGFR-mediated tumorigenesis via ERK-dependent upregulation of Nrp1, Flt1 and VEGF expression

which leads to autocrine tumor cell proliferation. Data represent mean ± SEM. * p≤0.05; ** p≤0.005; *** p≤0.0005.

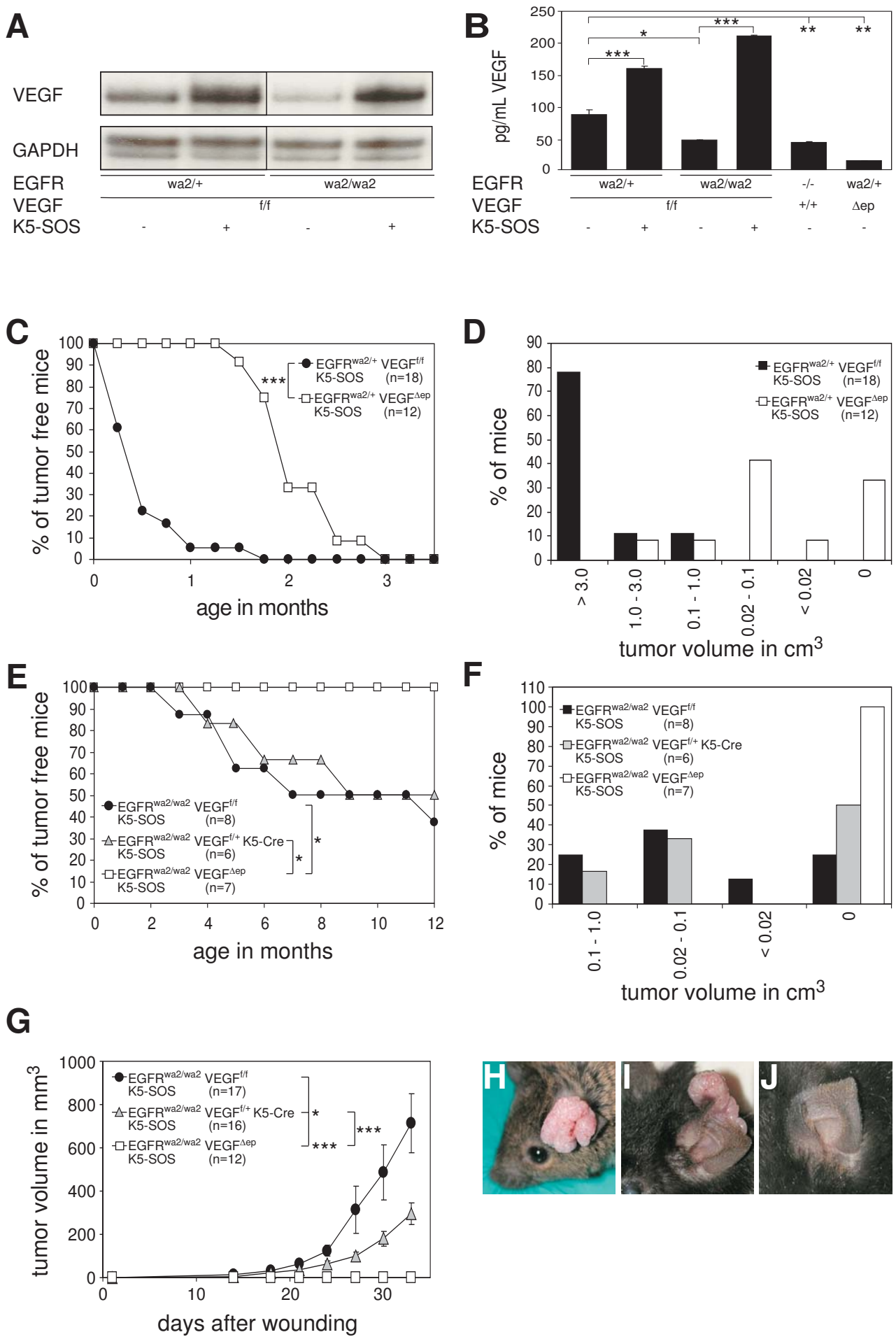


Fig.1; Lichtenberger et al.

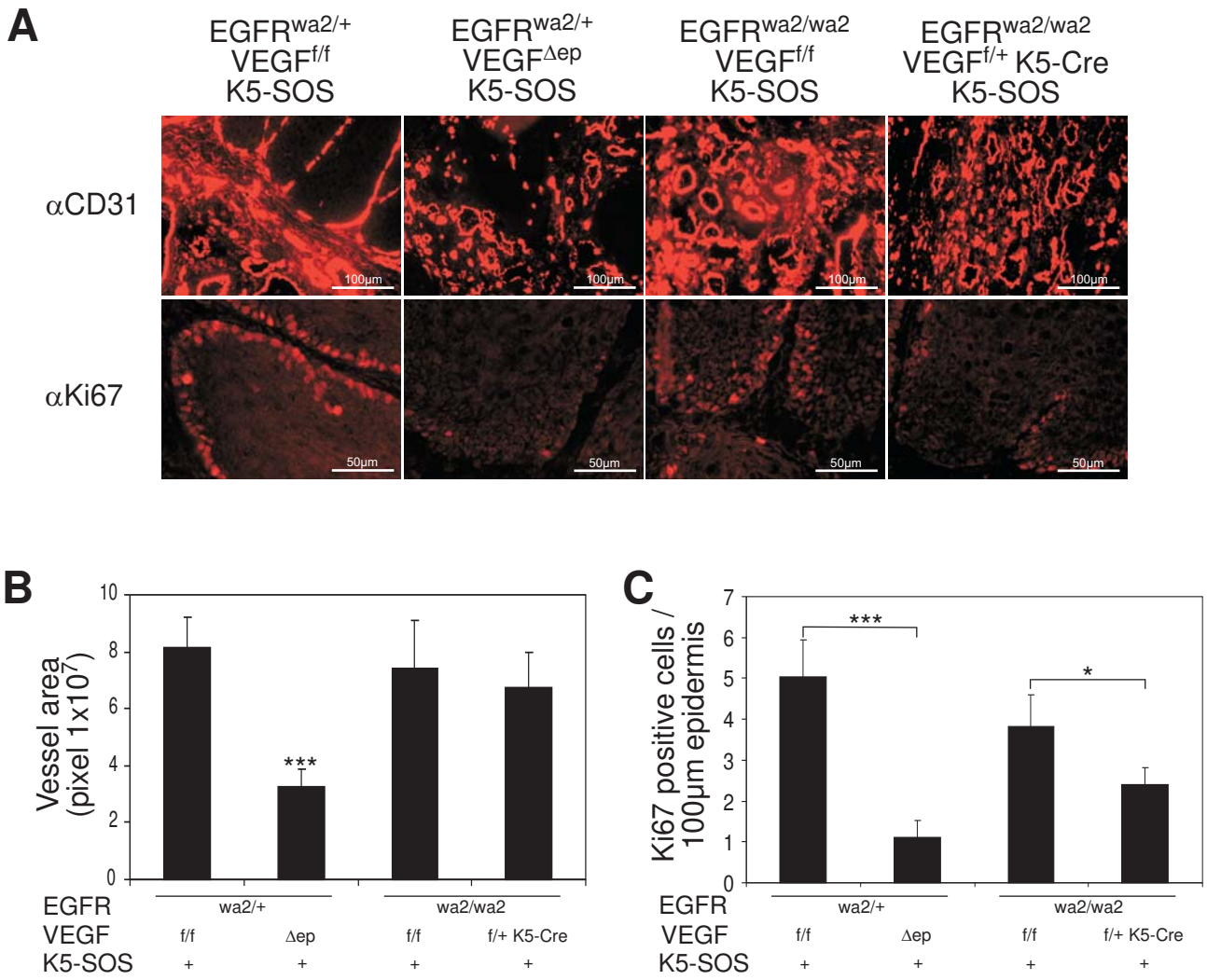


Fig.2; Lichtenberger et al.

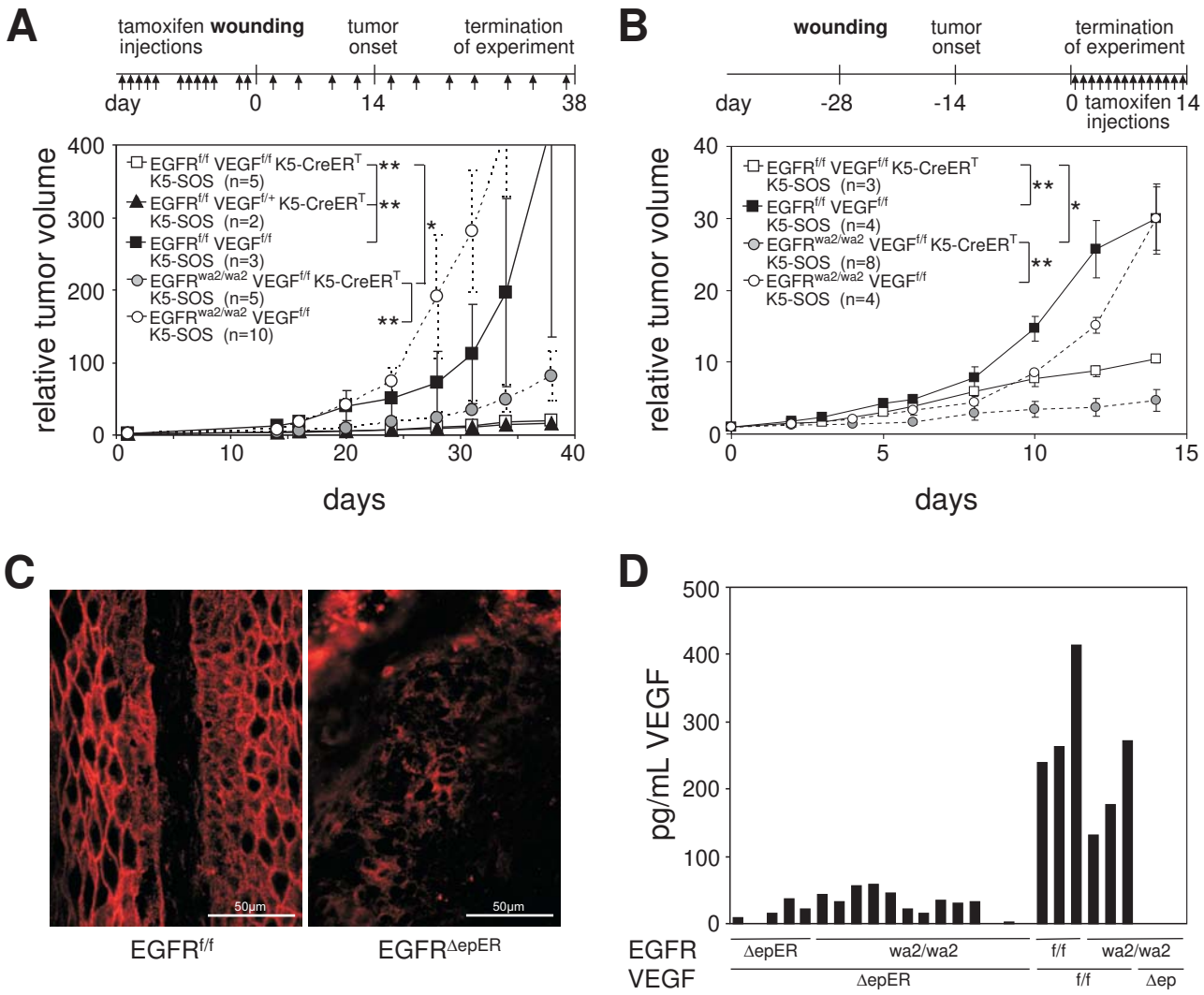
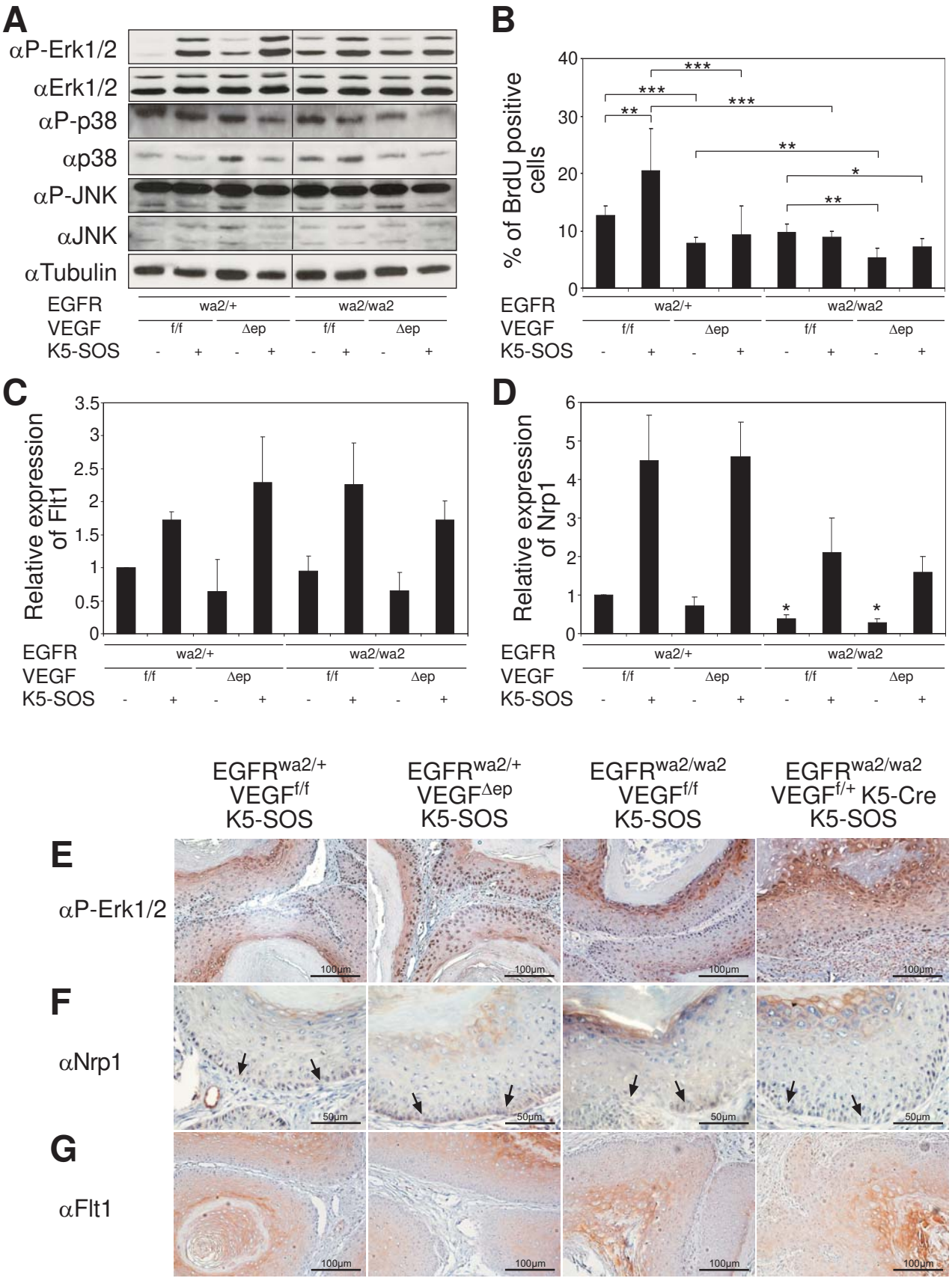


Fig.3; Lichtenberger et al.



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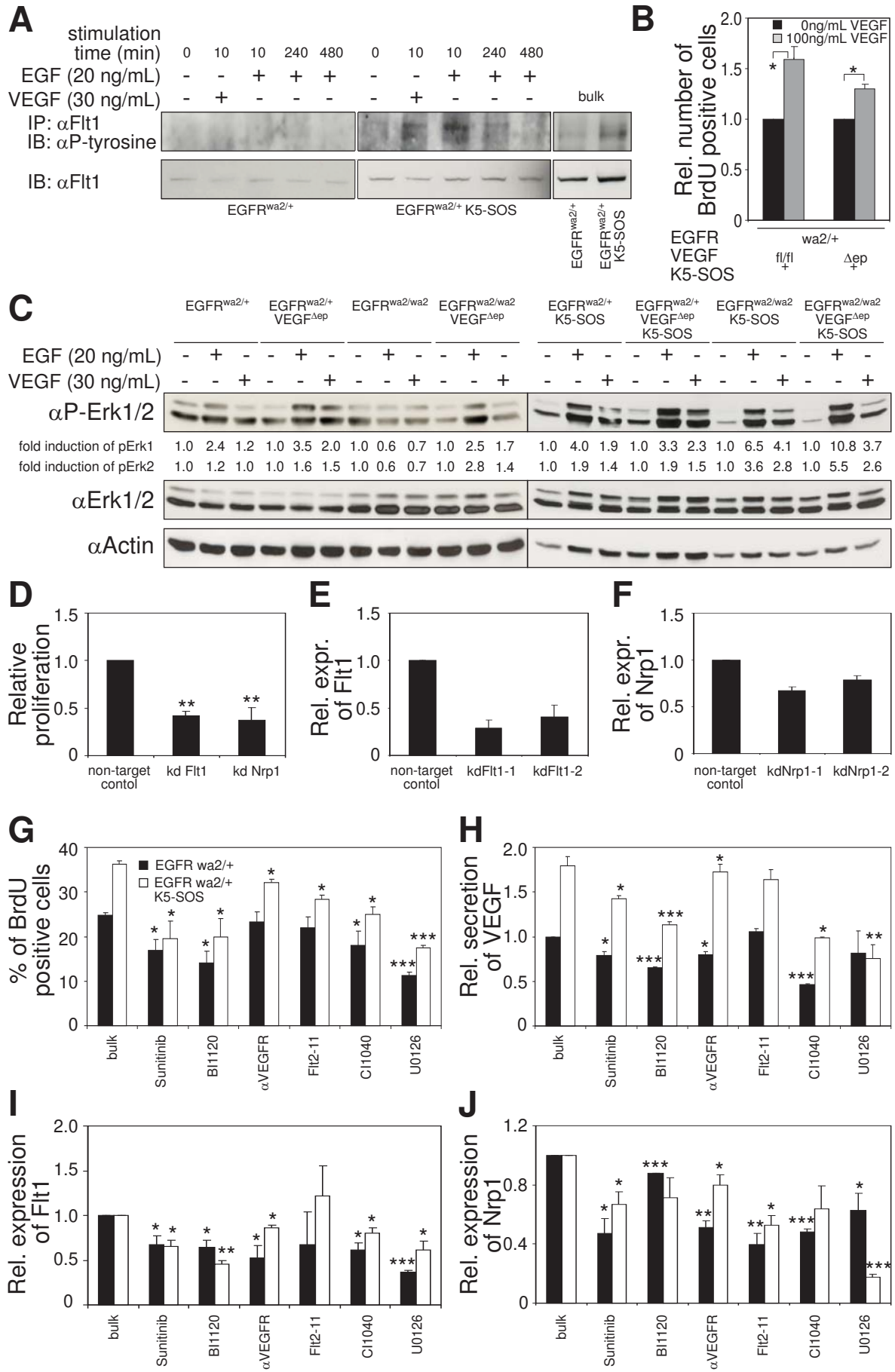


Fig.5; Lichtenberger et al.

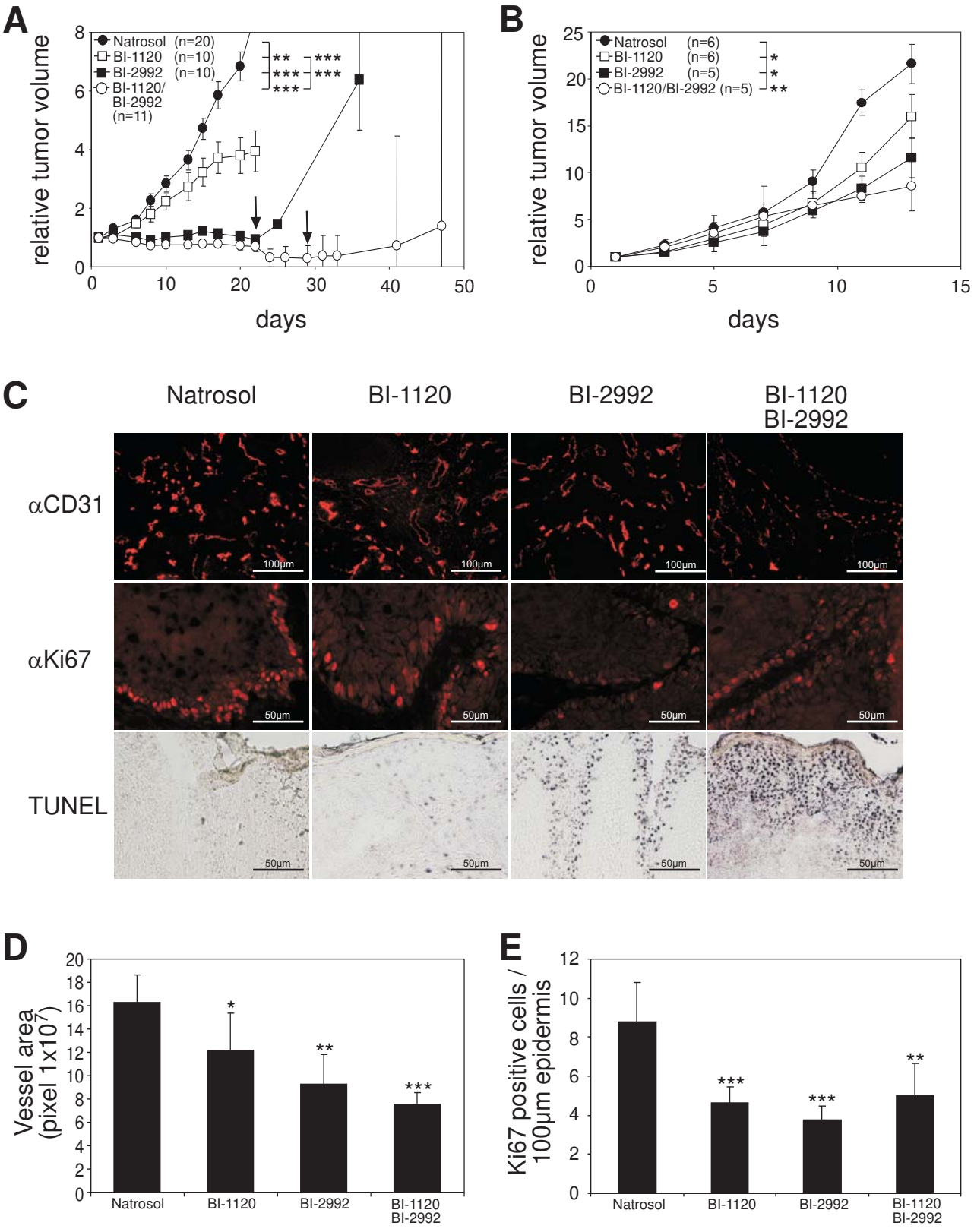


Fig.6; Lichtenberger et al.

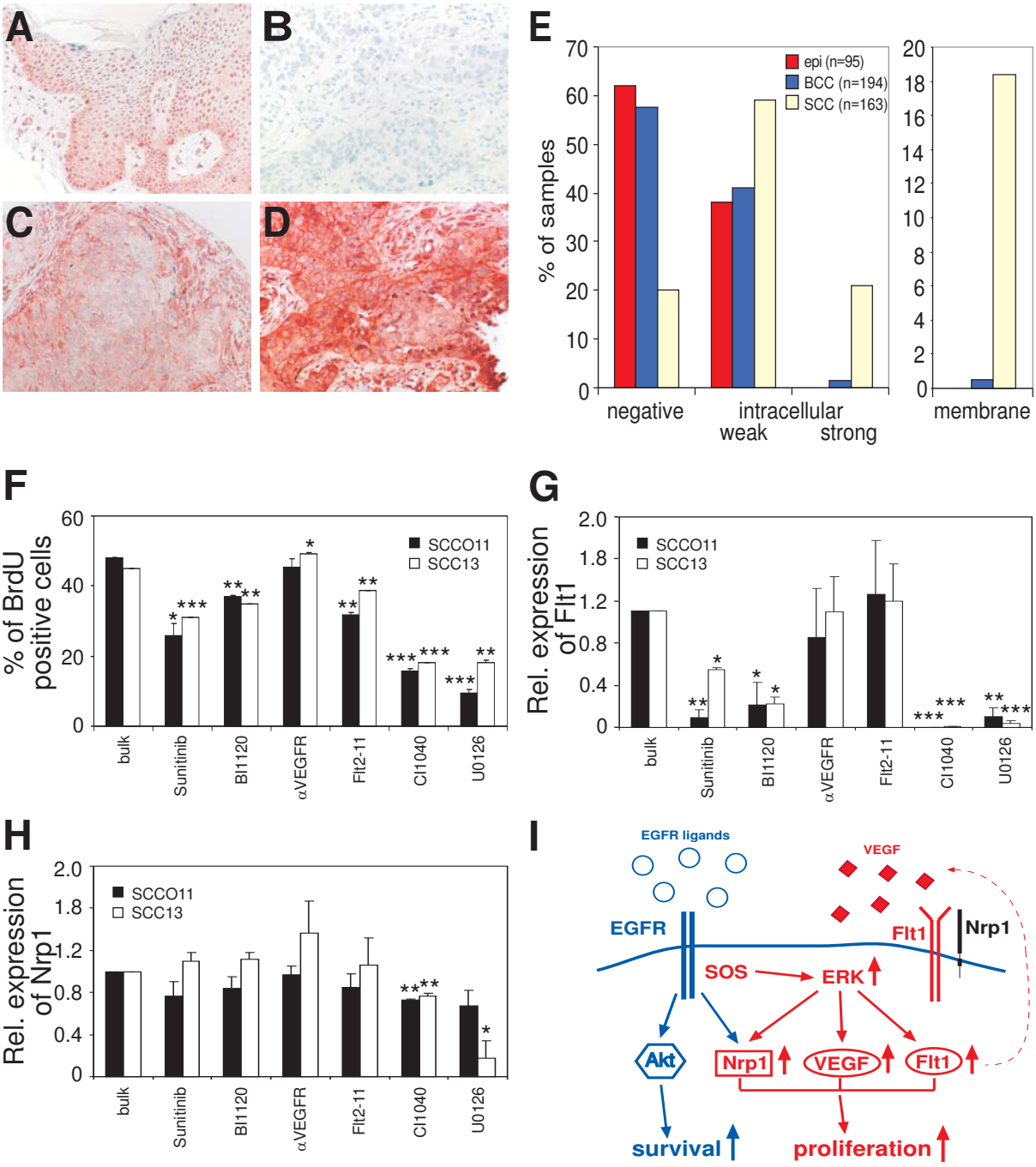
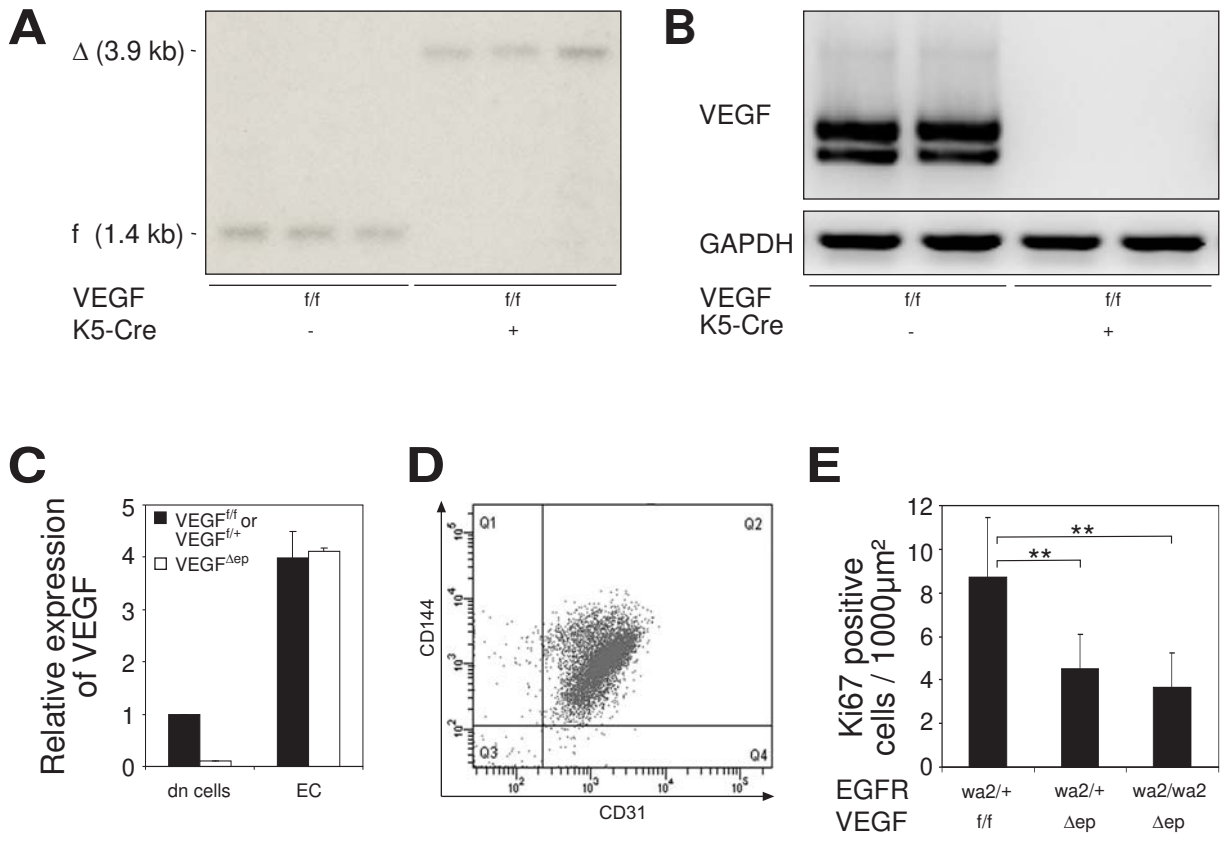
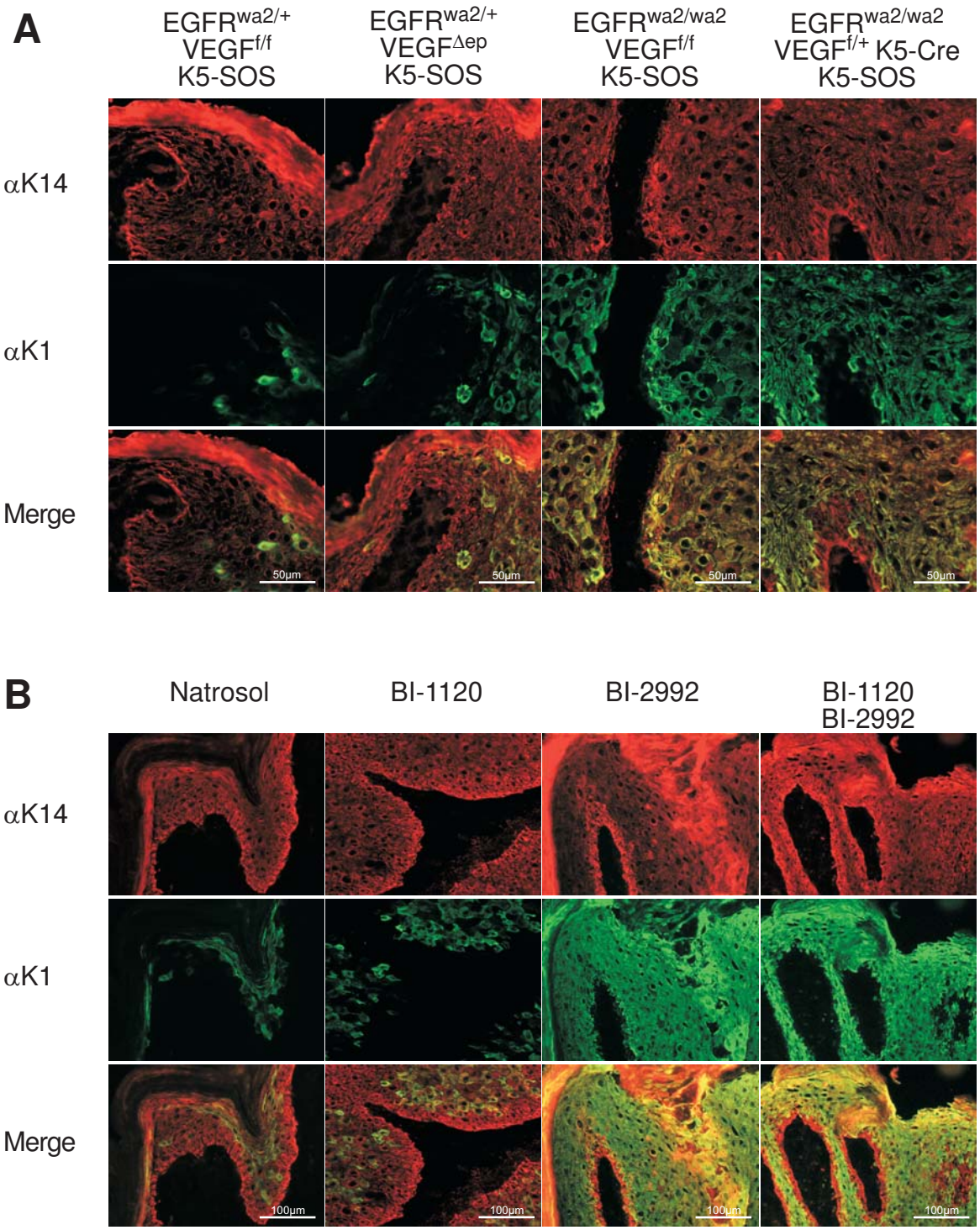


Fig.7; Lichtenberger et al.



Suppl. Fig.1; Lichtenberger et al.



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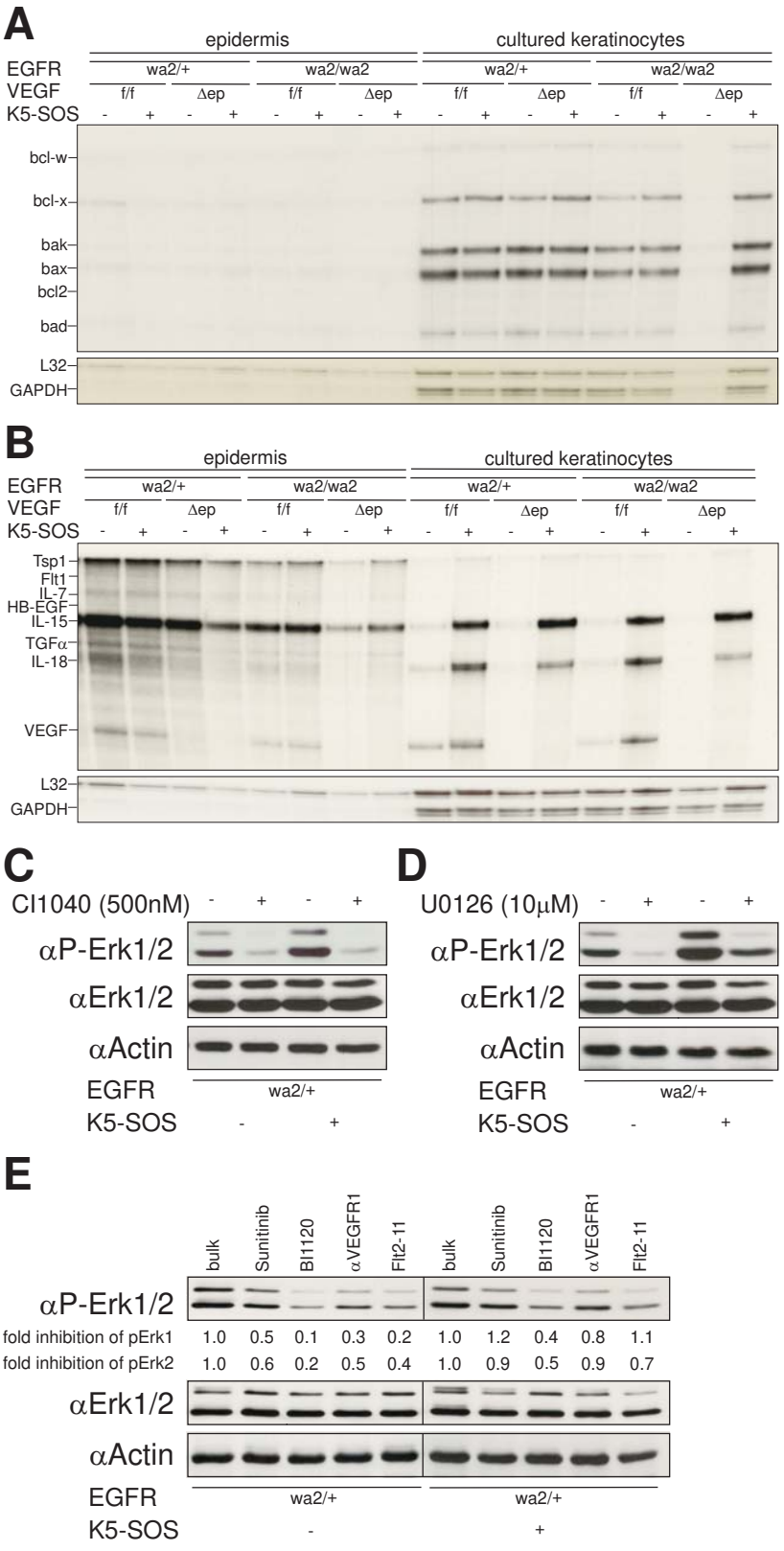

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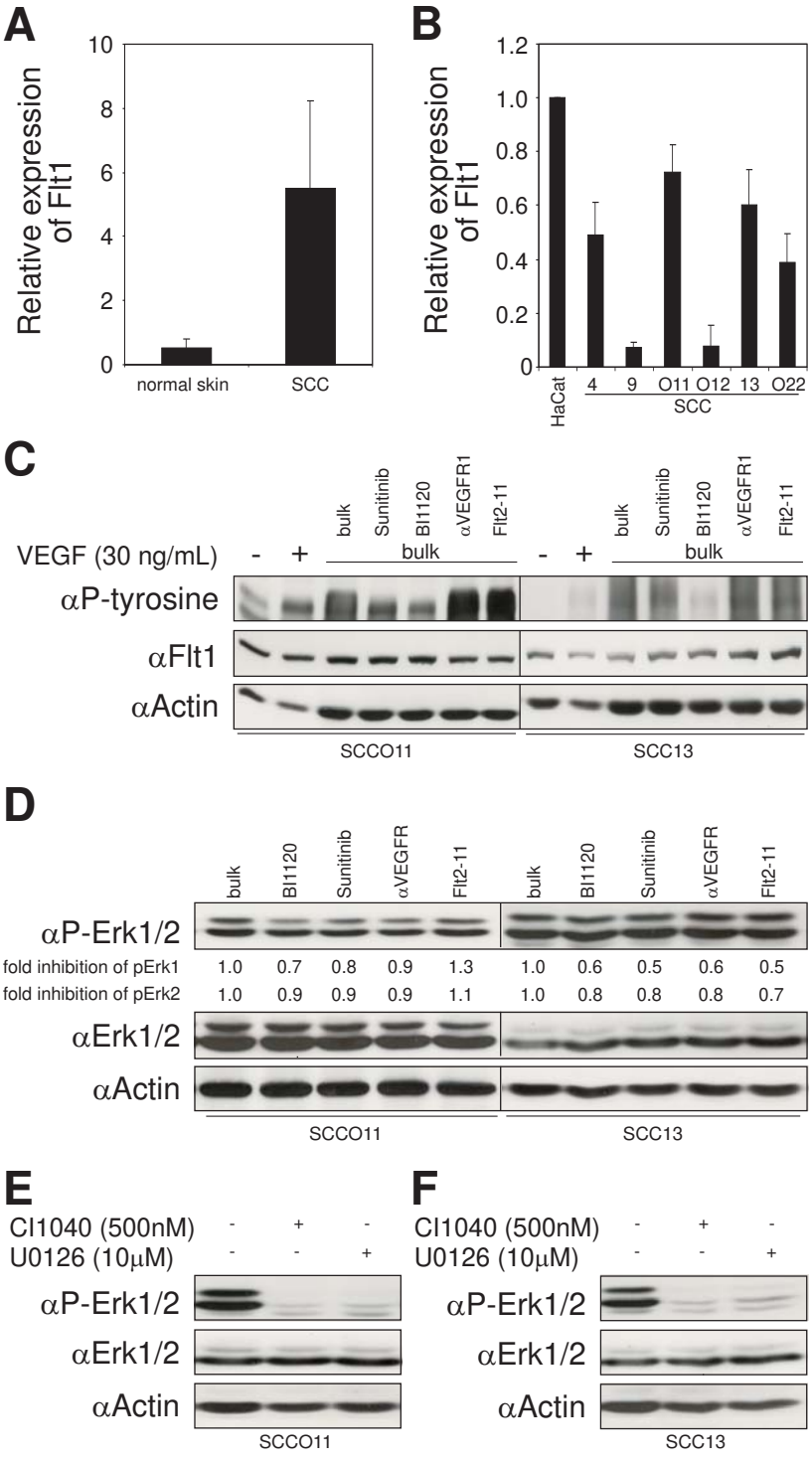

Suppl. Fig. 4; Lichtenberger et al.

Supplementary Figure 1

(A) Southern blot analysis of keratinocyte DNA isolated from mice of the indicated genotypes showing the non-recombined VEGF floxed allele and the cre-recombined Δ allele. **(B)** Semiquantitative real-time PCR showing complete absence of mRNA transcription of all VEGF splicing isoforms in keratinocytes isolated from $VEGF^{\Delta ep}$ mice. **(C)** qRT-PCR measuring the expression of VEGF in FACS-sorted $CD31^+$ $CD144^+$ endothelial cells and double negative cells from wild-type and $VEGF^{\Delta ep}$ mice. **(D)** Representative Dotplot showing FACS-sorted $CD31^+$ $CD144^+$ endothelial cells. **(E)** Quantification of Ki67 positive cells on epidermal sheets isolated from mice of the indicated genotypes. Data represent mean ± SEM of three independent samples. ** $p \leq 0.005$.

Supplementary Figure 2

(A) Immunofluorescent stainings with antibodies against keratin 14 and keratin 1 of sections of spontaneous papillomas from $EGFR^{wa2/+}$ K5-SOS and $EGFR^{wa2/+}$ K5-SOS $VEGF^{\Delta ep}$ mice and of wounding-induced tumors from $EGFR^{wa2/wa2}$ K5-SOS and $EGFR^{wa2/wa2}$ $VEGF^{f/+}$ K5-Cre K5-SOS mice. **(B)** Immunofluorescent staining on sections of K5-SOS papillomas treated with the indicated inhibitors.

Supplementary Figure 3

(A,B) RNAse protection assay showing mRNA expression levels of the indicated genes. RNA was isolated from epidermis or from cultured primary keratinocytes of the indicated genotypes **(C,D).** Western blot analysis showing reduction of ERK phosphorylation in keratinocytes of the indicated genotypes after treatment with the ERK inhibitors CI-1040 and UO126. **(E)** Western blot analysis showing phospho-ERK1/2 levels in keratinocytes after treatment with the VEGFR inhibitors Sunitinib (1μM), BI-1120 (500nM), an αVEGFR antibody (2,5μg/mL) and the VEGF blocking-

peptide Flt_{2-11} (1µg/mL). Numbers indicate the levels of Erk1/2 activation relative to the respective controls after correction with total ERK normalized to actin (loading control).

Supplementary Figure 4

(A,B) qRT-PCR measuring the expression of Flt1 in human skin cancer biopsies (n=5) compared to healthy skin (n=3; A) and in different human SCC cell lines (B). **(C)** Western Blot analysis showing the levels on phosphorylated tyrosine in VEGF-stimulated and bulk SCC cells treated with the VEGFR inhibitors Sunitinib (1µM), BI-1120 (500nM), an αVEGFR antibody (2,5µg/mL) and the VEGF blocking-peptide Flt_{2-11} (1µg/mL). **(D)** Western blot analysis showing phospho-ERK1/2 levels in SCCO11 and SCC13 cells after treatment with the indicated inhibitors. Numbers indicate the levels of Erk1/2 activation relative to the respective controls after correction with total ERK normalized to actin (loading control). **(E,F)** Western blot analysis showing reduced ERK phosphorylation in SCCO11 (E) and SCC13 (F) cells after treatment with the ERK inhibitors CI-1040 and UO126.

5.3 MANUSCRIPT: Conditional deletion of β1 integrin impairs SOS-dependent skin tumor development in transgenic mice

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ABSTRACT

There are several *in vitro* evidences for a crosstalk between the EGFR and β1 integrin, both of which are required for normal skin development and are also implicated in tumor formation. Transgenic mice expressing a dominant form of Son of Sevenless in basal keratinocytes (K5-SOS) develop spontaneous skin papillomas in the presence of a functional EGFR, but tumor formation is impaired in a hypomorphic (wa2) background. In this study we investigate the contribution of β1 integrin to SOS-induced tumors. Cultured keratinocytes isolated from K5-SOS transgenic mice exhibited elevated protein levels of Src and β1 integrin and showed enhanced migration and a reduced number of focal contacts after wounding. To examine whether high levels of β1 integrin contribute to tumor formation, we crossed K5-SOS transgenic mice to mice carrying conditional β1 integrin alleles and deleted β1 integrin in basal keratinocytes via K5-Cre. Tumor formation in these mice is blocked and immunoblotting analysis of downstream signaling effectors of β1 integrin and the EGFR revealed reduced phosphorylation of the focal adhesion kinase (FAK) and MAP kinases. Using a tamoxifen-inducible K5-CreER^{T} transgenic line we could also show delayed tumor growth upon tamoxifen treatment. These results suggest a crucial role for β1 integrin in tumor initiation as well as tumor progression and maintenance *in vivo*.

INTRODUCTION

The epidermis is a stratified squamous epithelium which consists of multiple layers of keratinocytes. Proliferation of keratinocytes takes place in the basal layer of the epidermis. Cells that are commited to undergo terminal differentiation withdraw from the cell-cycle and migrate upward to the suprabasal layers. The outermost layers are exquisitely specialized keratinocytes forming a protective barrier between the body and the environment and are composed of anucleated dead squames that are continuously shed from the surface of the skin. Keratinocyte proliferation and differentiation are tightly coordinated processes which are regulated by many factors including the epidermal growth factor receptor (EGFR) and integrins.

The EGFR/ErbB1/HER1 is the prototype of a family of receptor tyrosine kinases (RTK) consisting of four members, including ErbB2/Neu/HER2, ErbB3/HER3 and ErbB4/HER4. Binding of various ligands such as epidermal growth factor (EGF), transforming growth factor α (TGFα), amphiregulin, heparin-binding EGF (HB-EGF), betacellulin, epigen and epiregulin induces receptor homo- or heterodimerization, activation of the intrinsic tyrosine kinase and autophosphorylation of key tyrosines at the carboxy-terminal tail of the receptor, which in turn act as docking sites for multiple signaling proteins containing Src homology 2 (SH2) domains. Signaling molecules which were shown to directly interact with the EGFR include PLCγ, Shc, GTPase-activating protein (GAP) and growth factor receptor-bound protein (Grb2) (Fantl et al., 1993; Pawson, 1994; Schlessinger, 1994). One of the best characterized pathways activated by the EGFR is the Ras pathway. The adaptor protein Grb2 binds phosphorylated tyrosines at the C-terminus of the activated receptor and interacts via its SH3 domain with the Ras nucleotide exchange factor Son of Sevenless (SOS), thereby recruiting SOS to the plasma membrane (Schlessinger, 1994; Weiss et al., 1997). SOS facilitates GDP-GTP exchange and catalyses the activation of Ras, which in turn activates the cytoplasmic serine/threonine protein kinase Raf by recruiting it to the cell membrane (Marais et al., 1995). Raf phosphorylates the mitogen-activated

protein kinase/extracellular signal-regulated kinase (MAPK/ERK; MEK) proteins, and these activate the ERK subgroup of MAPKs. Activated ERKs translocate to the nucleus and lead to the induction of AP-1 target genes (Schlessinger and Ullrich, 1992). As shown previously, mice expressing an activated form of Son of Sevenless under the keratin 5 promoter (K5-SOS) develop skin papillomas at 100% penetrance in a wild-type EGFR background, and papilloma formation is impaired but can be induced by wounding in a hypomorphic (waved-2; wa2) EGFR background. In this system the EGFR provides an essential survival signal to epidermal tumor cells (Sibilia et al., 2000). The EGFR is overexpressed in a broad range of human cancers including glioblastoma and cancers of the breast, prostate, ovary, liver, bladder, esophagus, larynx, stomach, colon, lung and tumors of the head and neck (Nicholson et al., 2001; Salomon et al., 1995; Sharma et al., 2007; Sibilia et al., 2007).

Integrins are heterodimeric cell surface receptors consisting of α and β subunits that mediate the attachment of cells to the extracellular matrix (ECM) at sites called focal adhesions. Epidermal keratinocytes express several integrin receptors, including $\alpha2\beta1$, $\alpha3\beta1$ and $\alpha6\beta4$. Integrin engagement and subsequent clustering of these receptors activates multiple signaling pathways that affect actin cytoskeleton organization, cell adhesion and migration, proliferation, programmed cell death, tissue organization and differentiation. Integrins have been shown to interact with various signal-transducing components of focal adhesions, particularly the focal adhesion kinase (FAK) and c-Src, and they are also capable of activating the MAP kinases Erk1 and Erk2. It has also been reported that $\beta1$ integrin and MAPK cooperate to maintain the epidermal stem cell compartment (Zhu et al., 1999). FAK has been shown *in vitro* to bind the cytoplasmic tail of $\beta1$ integrin via its amino-terminal domain, and SH2 and SH3 domains of other focal adhesion proteins via its carboxy-terminal domain. Upon integrin-mediated activation FAK undergoes autophosphorylation at tyr^{397}, creating a high affinity binding site for the SH2 domain of Src, which in turn triggers transphosphorylation of FAK at

tyr^{576}, tyr^{577} and tyr^{925}, rendering FAK a fully active kinase. Phosphorylation at tyr^{925} allows interaction of FAK with the Grb2-SOS complex and subsequent activation of the MAPK pathway (Mitra et al., 2005; Schlaepfer and Hunter, 1997). Integrin engagement and clustering also leads to increased intracellular Ca^{2+} levels (Schwartz, 1993), intracellular pH (Schwartz et al., 1989; Schwartz et al., 1990), inositol lipid synthesis (McNamee et al., 1993) and expression of cyclins (Guadagno et al., 1993). Integrins are known to mediate invasion and metastasis (Guo and Giancotti, 2004), and a variety of human cancers have been linked to changes in expression and activation of integrins and integrin-coupled signaling effectors, including tumors of the skin, breast, colon, prostate and ovaries. Loss or upregulation of specific integrins can prevent squamous cell carcinoma (SCC) cells from undergoing apoptosis, and upregulation of integrin expression in differentiating keratinocytes can influence proliferation of stem cells in the underlying basal layer (Janes and Watt, 2006). β1 integrin expression has been shown to play an important role in the initiation and maintenance of mammary tumor growth as shown by disruption of β1 integrin function in the mammary epithelium of a transgenic mouse model of human breast cancer (White et al., 2004). FAK phosphorylation on tyr^{397} and tyr^{576} was found to be increased in aggressive uveal and cutaneous melanoma cells (Hess et al., 2005). Moreover, in a mouse skin carcinogenesis model ablation of FAK could reduce benign papilloma formation and inhibit progression to malignant squamous cell carcinoma (McLean et al., 2004).

The EGFR and β1 integrin are both expressed in proliferating keratinocytes of the basal layer of the epidermis and in hair follicles. There are several *in vitro* evidences for a crosstalk between the EGFR and β1 integrins, which might be mediated by signaling molecules like FAK, integrin-linked kinase (ILK), Shc, Src, and various adaptor proteins as well (Dedhar et al., 1999; Moro et al., 2002; Sieg et al., 2000). Integrins have been shown to associate with the EGFR and c-Src at cell membranes, thereby phosphorylating the EGFR (Moro et al., 2002). Similar to mice lacking β1 integrin in the

epidermis, mice deficient for the EGFR show defects in hair follicle development (Brakebusch et al., 2000; Sibilia et al., 2003; Sibilia and Wagner, 1995; Threadgill et al., 1995). In EGFR-deficient mice these defects can be partially rescued by epidermis-specific expression of SOS (Sibilia et al., 2000). Interestingly, mice with epidermis-specific deletion of β1 integrin display reduced expression of SOS in basal cells (unpublished data). Together, these results strongly suggest that a crosstalk between β1 integrin and EGFR signaling pathways might occur during skin development and tumor formation.

We have previously described the generation of transgenic mice expressing a dominant form of SOS (K5-SOS) under the control of a K5 promoter. In this study we demonstrate that keratinocytes isolated from K5-SOS transgenic mice express high levels of β1 integrin and Src and exhibit an enhanced migratory potential as well as a reduced number of focal contacts. To study the consequences of β1 integrin deletion in skin tumor initiation, maintenance and progression, we crossed K5-SOS transgenic mice to mice lacking β1 integrin expression in the epidermis generated either by crossing conditional β1 integrin mice to K5-Cre or tamoxifen-inducible K5-CreER^{T} transgenic lines. We found that tumor formation was inhibited if β1 integrin was deleted before tumor initiation and delayed if β1 integrin deletion occurred after tumors had developed. Moreover, we could relate this phenotype to downstream signaling effectors of β1 integrin and the EGFR. These findings demonstrate a pivotal role for β1 integrin in tumor initiation as well as maintenance and progression. Furthermore, they provide evidence for a crosstalk between the EGFR and β1 integrin *in vivo.*

RESULTS

Increased β1 integrin and Src protein levels in K5-SOS expressing keratinocytes

We have previously reported that transgenic mice expressing a dominant form of Son of Sevenless (K5-SOS) in basal keratinocytes develop skin papillomas at 100% penetrance. However, tumor formation is impaired in a hypomorphic EGFR (wa2) background (Sibilia et al., 2000). To investigate additional mechanisms leading to tumor formation primary keratinocytes from adult $EGFR^{wa2/+}$, $EGFR^{wa2/+}$ K5-SOS, $EGFR^{wa2/-}$, and $EGFR^{wa2/-}$ K5-SOS mice were isolated and the expression of several potential target molecules was investigated. Interestingly, we found that the protein levels of β1 integrin and Src were strongly increased in K5-SOS expressing keratinocytes, whereas FAK expression was unaffected (Fig. 1A). Increased levels of β1 integrin were also observed on the cell surface of K5-SOS expressing keratinocytes by flow cytometry (Fig. 1B).

It is known that the cytosolic tyrosine kinase Src is regulated by tyrosine phoshorylation mainly on tyr^{418} in the catalytic domain and on tyr^{529} in the C-terminus of the protein. Interestingly, when we stimulated growth factor starved keratinocytes with increasing doses of EGF no striking differences in tyrosine phosphorylation of Src could be observed suggesting that EGF stimulation and/or K5-SOS expression did not affect Src activation (Suppl. Fig. 1). However, since total protein levels of Src are increased in K5-SOS expressing epidermal cells but levels of pSrc [pY418] and pSrc [pY529] are comparable, these results might suggest that in K5-SOS transgenic keratinocytes the overall phosphorylation of Src is reduced compared to non-transgenic epidermal cells. Furthermore, we did not detect changes in the phosphorylation of tyr^{397} of FAK, a very important signaling molecule downstream of integrins which also recruits Src to sites of focal adhesions. These results show that K5-SOS expression in keratinocytes leads to increased β1 integrin and Src levels, which is independent of EGFR signaling.

K5-SOS transgenic epidermal cells display increased migration

Since β1 integrin and Src are known to be important for the formation and the downstream signaling of focal adhesions in keratinocytes we addressed if the increased protein of levels β1 integrin and Src found in K5-SOS transgenic epidermal cells affect the migratory potential of primary keratinocytes *in vitro*. Therefore, scratch wounds were applied to mitomycin C-treated keratinocyte cultures. Interestingly, the wounding experiments revealed a significant increase in the migration of keratinocytes expressing K5-SOS compared to non-transgenic cells (Fig. 2A-H,M). Furthermore, cell migration after wounding was impaired in hypomorphic $EGFR^{wa2/-}$ keratinocytes compared to $EGFR^{wa2/+}$ keratinocytes (Fig. 2E,G,M). K5-SOS expression could rescue the migration defect of $EGFR^{wa2/-}$ epidermal cells (Fig. 2G,H,M). However, the migration of $EGFR^{wa2/-}$ K5-SOS keratinocytes was delayed compared to $EGFR^{wa2/+}$ K5-SOS epidermal cells (Fig. 2F,H,M). To examine whether the increased Src protein levels in K5-SOS expressing keratinocytes are responsible for the increased migration of these cells, wounded keratinocytes were treated with the Src inhibitor SU6656. Interestingly, Src inhibition reverted the increased migration observed in K5-SOS expressing keratinocytes, and reduced migration was observed in Src inhibitor-treated $EGFR^{wa2/+}$ and $EGFR^{wa2/-}$ cells as well (Fig. 2E-M). These results demonstrate that K5-SOS transgenic keratinocytes display increased migration and that this occurs in a Src-dependent manner.

K5-SOS expressing keratinocytes display reduced numbers of focal contacts after wounding

Next we investigated if K5-SOS expression leads to changes in the organization of the actin cytoskeleton and the formation of focal adhesions after wounding. Immunostaining of migrating keratinocytes in the *in vitro* wounding assay revealed that the number of focal contacts was significantly reduced in $EGFR^{wa2/+}$ K5-SOS keratinocytes compared to wild-type controls, as evidenced by paxillin staining (Fig.

3A,B). In contrast, in EGFR$^{wa2/-}$ K5-SOS keratinocytes the reduction of focal contacts was not as strong compared to EGFR$^{wa2/-}$ cells (Fig. 3C,D). Moreover, a difference in the organization of actin in protrusive structures of the migrating keratinocytes could be detected. While we observed many filopodia in EGFR$^{wa2/+}$ K5-SOS keratinocytes (Fig. 3B), the number of these structures was clearly reduced in EGFR$^{wa2/-}$ K5-SOS keratinocytes (Fig. 3D), which was comparable to the phenotype of non-transgenic EGFR$^{wa2/+}$ and EGFR$^{wa2/-}$ epidermal cells (Fig. 3A,C). As shown in figure 2, the Src kinase-specific inhibitor SU6656 significantly reduced the elevated migratory potential of K5-SOS transgenic primary keratinocytes. Interestingly, increased numbers of focal contacts were observed in these cells upon SU6656 treatment (Fig. 3F,H), thus, indicating that total levels of Src play a crucial role in focal adhesion turnover. Moreover, β1 integrin also co-localized with paxillin at the sites of focal contacts (Fig. 3 I-P). However, we could not assess a difference in localization of β1 integrin within the cells of the investigated genotypes. These results demonstrate that the reduced number of focal contacts observed in EGFR$^{wa2/+}$ K5-SOS transgenic keratinocytes correlates with their increased migratory potential. These effects, which seem to depend on EGFR signaling, can be reverted by Src inhibition.

Since β1 integrin is upregulated in K5-SOS transgenic keratinocytes we investigated whether this might also have an impact on the adhesive capacity of these cells. Interestingly, we found reduced binding of hypomorphic EGFR$^{wa2/-}$ keratinocytes to collagen I, collagen IV and to laminin when compared to wild-type epidermal cells (Suppl. Fig. 2). While we found no difference in adhesion between EGFR$^{wa2/+}$ and EGFR$^{wa2/+}$ K5-SOS keratinocytes, K5-SOS expression could rescue the adhesion defect of keratinocytes in the hypomorphic EGFR background (Suppl. Fig. 2). Furthermore, only weak binding to fibronectin and vitronectin was observed in all keratinocytes (Suppl. Fig. 2).

Molecular analysis of signaling pathways

Next we addressed the activation of downstream signaling pathways and cell cycle associated proteins. When we looked at the basal activation of MAPK and Ras, we found a significant increase in phosphorylation levels of Erk1 and Erk2 in K5-SOS transgenic keratinocytes compared to non-transgenic controls (Fig. 4A), but interestingly, not in Ras-GTP levels (Fig. 4C). This might indicate that K5-SOS leads to a constitutive activation of the MAPK pathway in these cells. Inhibition of Src via the inhibitor SU6656 did not affect Erk1/2 phosphorylation in any of the investigated genotypes. It has been reported that Erk5 signals downstream of Src (Abe et al., 1997; Scapoli et al., 2004). Given that SOS is a GEF for Ras and due to the finding that the expression of K5-SOS in primary keratinocytes leads to an increase in the Src protein level, we wanted to examine the effect of K5-SOS on EGF-dependent activation of Erk5. We found a strong increase in the phosphorylation of Erk5 in response to EGF stimulation both in $EGFR^{wa2/+}$ and $EGFR^{wa2/+}$ K5-SOS keratinocytes, but interestingly not in the EGFR hypomorphic background, neither in $EGFR^{wa2/-}$ nor in $EGFR^{wa2/-}$ K5-SOS keratinocytes (Fig. 4B). The degree of Erk5 phosphorylation upon EGF stimulation was comparable between $EGFR^{wa2/+}$ and $EGFR^{wa2/+}$ K5-SOS keratinocytes, indicating that phosphorylation of Erk5 is independent of the expression of SOS but rather depends on the EGFR. Like Erk1/2, phosphorylation levels of Erk5 were also not affected by Src inhibition (data not shown), therefore excluding that phosphorylation of Erk proteins occurs in a Src-dependent manner.

Interestingly, when we looked at cell cycle associated proteins $EGFR^{wa2/-}$ keratinocytes showed a clear reduction in the levels of Cdk1 and p27, and also to a lower extent of Cdk2, Cdk4 and Cyclin D1, which was completely rescued in $EGFR^{wa2/-}$ K5-SOS epidermal cells (Fig. 4D). However, the expression of Cdk1, Cdk2, Cdk4, p27 and Cyclin D1 was similiar in keratinocytes isolated from $EGFR^{wa2/+}$, $EGFR^{wa2/+}$ K5-SOS, and $EGFR^{wa2/-}$ K5-SOS mice. Expression levels of p21 remained unaffected in keratinocytes of the different genotypes. These results suggest that the K5-SOS

transgene affects migration of primary keratinocytes in a Src-dependent manner, most likely due to enhanced activation of Erk1/2 MAP kinases.

Lack of β1 integrin impairs skin tumor development in K5-SOS transgenic mice

Mice expressing the K5-SOS transgene under the K5 promoter form big tumors at 100% penetrance in a wild-type EGFR background (Sibilia et al., 2000). To investigate whether β1 integrin deletion affects K5-SOS-dependent tumor formation, we crossed K5-SOS transgenic mice to mice carrying conditional (floxed) β1 integrin alleles ($\beta1int^{f/f}$) and deleted β1 integrin in the epidermis and tumors by employing K5-Cre transgenic mice ($\beta1int^{\Delta ep}$).

K5-SOS expression dramatically exacerbated the phenotype of $\beta1int^{\Delta ep}$ mice (Fig. 5A-C). Whereas in the first postnatal days the phenotype of $EGFR^{wa2/+}$ $\beta1int^{\Delta ep}$ K5-SOS mice was similar to the phenotype described for $\beta1int^{\Delta ep}$ mice (Brakebusch et al., 2000), around day 5 after birth $EGFR^{wa2/+}$ $\beta1int^{\Delta ep}$ K5-SOS were clearly distinguishable from $\beta1int^{\Delta ep}$ littermates without transgene, and skin abnormalities developed much faster (Fig. 5A). Around postnatal day 13 $EGFR^{wa2/+}$ $\beta1int^{\Delta ep}$ K5-SOS transgenic mice displayed severe cachexia, completely stopped growing (Fig. 5E) and did not survive longer than 4 weeks. Interestingly, $EGFR^{wa2/+}$ $\beta1int^{\Delta ep}$ K5-SOS mice developed no tumors, whereas many of the $EGFR^{wa2/+}$ K5-SOS mice had already developed tumors within the first 2 weeks as previously described (Fig. 5D,F) (Sibilia et al., 2000).

From the above experiments we conclude that tumors can not develop in K5-SOS transgenic mice in the absence of β1 integrin, suggesting that β1 integrin is needed for tumor initiation.

To investigate if β1 integrin is also needed for tumor maintenance and progression we crossed $EGFR^{wa2/+}$ $ß1int^{f/f}$ K5-SOS mice with a tamoxifen-inducible $K5\text{-}CreER^{T}$ transgenic line. After tumors had developed, mice were treated with tamoxifen for 11 days and tumor development was monitored. Interestingly, deletion of β1 integrin

significantly delayed tumor growth when compared to EGFR$^{wa2/+}$ K5-SOS control mice (Fig. 5G). These results reveal that K5-SOS-induced tumors fail to form in β1int$^{\Delta ep}$ mice and that tumor growth is delayed when β1 integrin is deleted in mice with existing tumors. Moreover, these findings provide evidence that β1 integrin is required for tumor initiation as well as tumor maintenance and progression.

Hyperthickened epidermis and perturbed expression of keratins in K5-SOS transgenic mice lacking β1 integrin

Upon deletion of ß1 integrin the architecture of the skin seems to undergo profound changes. It has previously been reported that the epidermis of β1int$^{\Delta ep}$ mice is thickened and shows perturbed expression of keratins 1 and 14, as the proportion of suprabasal, terminally differentiating keratinocytes is increased from 20 to 40% (Fig. 6A,B,I,J) (Brakebusch et al., 2000) probably because of reduced proliferation. This effect is even stronger in the presence of the K5-SOS transgene (Fig. 6B,D,J,L). Hematoxylin and Eosin staining on skin sections revealed that the epidermis of EGFR$^{wa2/+}$ β1int$^{\Delta ep}$ K5-SOS mice is more than twice as thick as the epidermis of EGFR$^{wa2/+}$ β1int$^{\Delta ep}$ littermates (Fig. 6B,D). However, this hyperplastic condition did not resemble the histological appearance of EGFR$^{wa2/+}$ K5-SOS tumors, which display characteristic features of papillomas (Fig. 6C). Immunofluorescent stainings for K14 and K1 on backskin of EGFR$^{wa2/+}$ β1int$^{\Delta ep}$ and EGFR$^{wa2/+}$ β1int$^{\Delta ep}$ K5-SOS mice showed that the basal, K14 expressing layer of the epidermis consists of multiple cell layers (Fig. 6E-H,M-P), and there is also an increase in the number of terminally differentiated, suprabasal cell layers expressing K1 (Fig. 6I-P) when compared to EGFR$^{wa2/+}$ β1int$^{\Delta ep}$ skin. Staining for β1 integrin revealed that integrin expression is upregulated in EGFR$^{wa2/+}$ K5-SOS tumors compared to expression levels in wild-type epidermis (Fig. 6Q-T).

Molecular analysis of K5-SOS transgenic epidermis lacking β1 integrin

Western blot analysis revealed that the levels of β1 integrin were increased in protein lysates prepared from the epidermis of K5-SOS transgenic mice (Fig. 7A), which confirms the data obtained by immunofluorescent staining (Fig. 6S). Interestingly, at the mRNA level no increase in β1 integrin expression could be observed in K5-SOS transgenic epidermis compared to non-transgenic controls, suggesting that integrin turnover and degradation are negatively regulated by K5-SOS expression (Fig. 7B). At the age of 3 weeks, ß1 integrin is almost completely absent at the protein as well as mRNA level in $EGFR^{wa2/+}$ $\beta1int^{\Delta ep}$ and $EGFR^{wa2/+}$ $\beta1int^{\Delta ep}$ K5-SOS mice, demonstrating that K5-Cre-mediated deletion of β1 integrin is very efficient (Fig. 7A,B).

An increase in the levels of total Src, as observed in plated keratinocytes, could not be seen in the epidermis of K5-SOS transgenic mice (Fig. 7A). Src levels were similar in epidermal lysates isolated from mice of all genotypes, indicating that Src upregulation most likely occurs exclusively in plated keratinocytes. Accordingly, the lack of ß1 integrin had no impact on the phosphorylation of the main regulatory sites [pY418] and [pY529] as well as on the total levels of Src (Fig. 7A).

Although the total protein levels of FAK remained unaffected in K5-SOS transgenic mice irrespective of β1 integrin expression, deletion of β1 integrin affects the phosphorylation of various tyrosine residues of FAK. Phosphorylation of tyrosines 397, 407, 576 and 861 of FAK was clearly reduced in β1 integrin deficient epidermis (Fig. 7C). Interestingly, also in epidermis isolated from $EGFR^{wa2/+}$ $\beta1int^{\Delta ep}$ K5-SOS mice, which do not develop papillomas, phosphorylation of tyr^{397} and tyr^{576} of FAK was clearly reduced compared to epidermis of wild-type or K5-SOS transgenic mice (Fig. 7A), suggesting that impaired FAK activation is responsible for the inhibition of tumor development in $EGFR^{wa2/+}$ $\beta1int^{\Delta ep}$ K5-SOS mice. Next, we investigated MAP kinase activation in the epidermis. Although we could not detect a significant difference in levels of phospho-Erk5 in the epidermis of the indicated genotypes (Fig. 7A),

phosphorylation of Erk1/2 was clearly reduced in EGFR$^{wa2/+}$ β1int$^{\Delta ep}$ K5-SOS mice (Fig. 7A). These results provide a mechanistic explanation that integrin-triggered activation of FAK and Erk1/2 is required for tumor formation. It seems that in the absence of β1 integrin the complex interplay between FAK and Erk is disturbed, and since Erk1 and 2 are not only implicated in the turnover of focal adhesions but also in the induction of cell proliferation, this might provide a second mechanism by which tumor initiation and maintenance are controlled.

DISCUSSION

K5-SOS transgenic mice develop spontaneous skin papillomas in a wild-type EGFR background and tumor formation can be induced by wounding in a hypomorphic EGFR background (Sibilia et al., 2000). Primary keratinocytes isolated from K5-SOS transgenic mice and plated onto collagen/fibronectin coated culture dishes show elevated protein levels of β1 integrin and Src, but not of focal adhesion kinase (FAK), leading to the question whether the adhesive or migratory potential of these cells might be affected.

Indeed, we could demonstrate that in K5-SOS transgenic keratinocytes migration is increased and the number of focal contacts is reduced upon wounding. In addition, treatment with the Src kinase-specific inhibitor SU6656 can revert this effect. While phosphorylation of the Src regulatory sites tyr^{418} and tyr^{529} was not altered in K5-SOS transgenic epidermal cells, the number of total Src molecules was increased. Moreover, in cultured keratinocytes phosphorylation of the FAK autophosphorylation site tyr^{397} was not affected by K5-SOS transgene expression.

Cultured keratinocytes of K5-SOS transgenic mice also displayed enhanced phosphorylation of Erk1/2 MAP kinases. Interestingly, Ras-GTP levels were not constitutively elevated in K5-SOS transgenic epidermal cells. Thus, it remains largely unclear how Erk1/2 kinases are activated. Integrin signaling to Erk is thought to occur via 2 different pathways. The Shc pathway, activated by α subunits of some integrins, transduces signals via Grb2-SOS/ Ras/Raf-1/MEK, whereas the FAK pathway, which is activated by most integrins, signals through Src/CAS/Crk-C3G/Rap-1/B-Raf/MEK to activate Erk (Barberis et al., 2000). Since in K5-SOS transgenic keratinocytes Src and ß1 integrin levels, but not Ras-GTP levels are elevated, Erk is likely activated via this second pathway.

Erk2 is known to play an important role in the formation and turnover of focal adhesions. Upon integrin engagement activated Erk2 is targeted to sites of focal assembly (Fincham et al., 2000) where it phosphorylates the myosin light chain kinase

(MLCK), thereby enhancing cellular migration (Klemke et al., 1997). Furthermore, Erk activation causes changes in gene expression that can promote migration.

Furthermore, It has previously been reported that Erk2 is part of a regulatory cycle of FAK/Src activation. On the one hand, Erk2 activation can promote FAK phosphorylation at ser^{910}, which subsequently leads to decreased paxillin binding to FAK and accelerated release of FAK from focal adhesions (Hunger-Glaser et al., 2004). On the other hand, Erk2-mediated phosphorylation of paxillin can facilitate FAK binding to paxillin and enhance FAK activation (Ishibe et al., 2003; Liu et al., 2002). Moreover, Erk2 is also required for the establishment of a calpain2/FAK/Erk2 complex promoting the proteolysis of FAK, focal adhesion turnover and accelerated cell migration (Carragher et al., 2003). Expression of K5-SOS stimulates Erk1/2 activation and causes higher β1 integrin protein levels. Therefore, it is possible that integrins, SOS and Erk cooperate in a positive feedback loop of mutual activation. Taken together, this could result in a faster turnover of focal adhesions and an enhanced migratory potential of K5-SOS transgenic keratinocytes.

In addition, we also found Erk5 activation in response to EGF-stimulation in cultured keratinocytes, but only in a wild-type EGFR background and independent of K5-SOS expression, which might suggest that the EGFR provides an essential stimulus for Erk5 to be activated. However, we could not detect a difference in Erk5 phosphorylation in the epidermis, neither in a hypomorphic EGFR background, nor upon β1 integrin deletion. These data also provide evidence that Erk5 activation occurs independently of Erk1/2 activation as previously described (English et al., 1999).

Interestingly, in a wild-type EGFR background the K5-SOS transgene had no effect on the adhesion capacity of keratinocytes to collagen I and collagen IV, as one would expect reduced adhesion as a result of a lowered number of focal contacts on these cells. It is possible that the increased levels of β1 integrin on the surface of K5-SOS transgenic keratinocytes compensate for this effect. EGFR hypomorphic keratinocytes displayed strongly reduced binding to collagen I and collagen IV compared to wild-type

cells. As previously reported, the EGFR inhibits keratinocyte differentiation and keeps basal cells in the proliferative compartment attached to the basal membrane (Sibilia et al., 2000). It seems that the lack of growth factor signaling induces cell detachment, probably because both, EGFR and integrin signaling are required for the maintenance of focal adhesions and the integrity of the actin cytoskeleton.

Keratinocytes in a hypomorphic EGFR background display reduced levels of Cdk1 and p27, and to a lower extent also of Cdk2, Cdk4 and Cyclin D1, all of which are cell cycle-associated proteins, and their expression was shown to be regulated by growth factors as well as by integrins. Expression of K5-SOS in $EGFR^{wa2/-}$ cells can rescue the reduced expression of these proteins, possibly by activating Erk. Activated Erk is known to induce the expression of Cyclin D1 and the Cdk inhibitory protein p21, which in turn inhibits the expression of cyclin E/Cdk2. However, sufficient Erk activation is only achieved when both, growth factor receptor and integrin signaling are fully active. Interestingly, growth factor signaling via RhoA is thought to inhibit expression of p27 (Danen et al., 2000), whereas in our case ablation of EGFR signaling seems to block expression of p27.

Interestingly, total levels of Src in freshly isolated epidermis of $EGFR^{wa2/+}$, $EGFR^{wa2/+}$ K5-SOS, $EGFR^{wa2/-}$ and $EGFR^{wa2/-}$ K5-SOS mice were comparable, indicating that elevated levels of Src protein in cultured keratinocytes isolated from K5-SOS transgenic mice are an *in vitro* stress response and do not contribute to the high tumorigenicity of these mice. However, β1 integrin levels remain elevated in epidermal protein lysates from K5-SOS transgenic mice. Therefore, it is likely that integrin signaling provides a potent stimulus for tumors to grow. However, since we did not detect a significant increase in β1 integrin mRNA transcription in the epidermis of K5-SOS transgenic mice, we speculate that higher integrin levels might be caused by reduced internalization and degradation of these proteins.

Importantly, expression of the K5-SOS transgene in β1int$^{\Delta ep}$ mice exacerbates the phenotype of these animals. K5-SOS expression causes even more severe changes to the skin architecture resulting in a 3-fold hyperthickening of the epidermis compared to EGFR$^{wa2/+}$ β1int$^{\Delta ep}$ mice. The most striking finding was that even though EGFR$^{wa2/+}$ β1int$^{\Delta ep}$ K5-SOS mice displayed enormous hyperthickening of the epidermis, they did not develop skin tumors. Moreover, tamoxifen–induced deletion of β1 integrin in mice that had already developed tumors, significantly delayed SOS-dependent tumor development, indicating that ß1 integrin is not only required for initiation of tumor growth, but also for their tumor maintenance and progression.

Molecular analysis of the epidermis of EGFR$^{wa2/+}$ ß1int$^{\Delta ep}$ and EGFR$^{wa2/+}$ β1int$^{\Delta ep}$ K5-SOS mice revealed a clear reduction in phosphorylation of FAK at various phosphorylation sites, including the autophosphorylation site tyr^{397} and the major activation site tyr^{576} , as well as a reduction in phospho-Erk1/2 levels. In contrast, phosphorylation of Src was not altered. It has previously been reported that an aggressive melanoma phenotype goes along with enhanced phosphorylation at these sites (Hess et al., 2005). FAK phosphorylation is reported to be altered in a variety of different tumors (McLean et al., 2005), affecting adhesion and migration of tumor cells. Furthermore, FAK signaling through the Erk/MAPK pathway has also been proposed to maintain growth in different tumor cells (Aguirre Ghiso, 2002). Conditional deletion of β1 integrin in mouse mammary epithelium dramatically impaired mammary tumorigenesis, and, interestingly, also in this model phosphorylation of FAK at tyrosines 397 and 576 was clearly reduced, whereas phosphorylation of Src remained unaffected (White et al., 2004). Moreover, epidermis-specific deletion of FAK suppressed chemically induced skin tumor formation (McLean et al., 2004). Since both FAK and Erk play important roles in the signaling network orchestrating migration, it is well possible that the reduced activity of these proteins following ablation of β1 integrin contributes to delayed or blocked tumor growth. Taken together, our findings

demonstrate a crucial role for β1 integrin in tumor initiation as well as maintenance and progression. Furthermore, they provide evidence for a crosstalk between the EGFR and β1 integrin *in vivo.*

MATERIALS AND METHODS

Generation of EGFR$^{wa2/+}$ β1int$^{\Delta ep}$ K5-SOS mice

The generation of K5-SOS transgenic mice and of mice carrying a conditional ß1 integrin gene was described previously (Potocnik et al., 2000; Sibilia et al., 2000). Mice carrying floxed ß1 integrin alleles (ß1int$^{f/f}$ mice) were crossed with either EGFR$^{wa2/wa2}$ K5-SOS transgenic mice or EGFR$^{wa2/wa2}$ K5-Cre mice (Tarutani et al., 1997). Offspring of these mice were then crossed to generate EGFR$^{wa2/+}$ ß1int$^{f/f}$ K5-Cre K5-SOS (EGFR$^{wa2/+}$ ß1int$^{\Delta ep}$ K5-SOS) mice in which ß1 integrin was deleted in the basal layers of the epidermis. For the temporal regulation of ß1 integrin deletion the tamoxifen-inducible K5-CreER^{T} transgenic line was employed (Indra et al., 1999). For inducible ß1 integrin deletion adult mice were injected intraperitoneally with 1mg of tamoxifen (Sigma; sunflower seed oil/ethanol mixture (10:1) at 10mg/mL) per day on 5 consecutive days and then twice a week for maintenance. Mice were kept in the animal facility of the Medical University of Vienna in accordance with institutional policies and federal guidelines.

Isolation and culture of mouse keratinocytes

Mouse keratinocytes were isolated as previously described and cultured on vitrogen-fibronectin-coated dishes in low calcium MEM medium containing 8% chelated FCS and grown at 32°C in a humidified 5% CO_2 incubator (Sibilia et al., 2000).

Migration and adhesion assay

Confluent monolayers of keratinocytes grown on 10cm culture dishes were treated with 1µg/mL Mitomycin C for 2 hours at 37°C and subsequently wounded with a yellow tip. Migration of cells was monitored in randomly determined fields over a period of 0-36h. Where indicated, Src-Inhibitor SU6656 was added at a concentration of 1µM. Cells which had migrated into the wound were counted at the indicated time-points. For the

adhesion assay, a Chemicon CytoMatrix Screening Kit was used according to the manufacturer's recommendations.

Immunofluorescence stainings

Keratinocytes cultured in slide flasks were wounded and at the indicated time-points fixed in 4% PFA, permeabilized with 0,1% Triton X-100/0,1 % sodium citrate for 2 minutes on ice, washed 2 times with PBS and blocked with 2% BSA/5% goat serum/PBS for 30 minutes at room temperature. Primary and secondary antibodies were diluted in 0,2% BSA/PBS, and applied according to standard procedures. The following antibodies were used: anti-β1 integrin (Chemicon), anti-Paxillin (BD Transduction), Alexa594-conjugated Phalloidin (Molucular Probes), and secondary Alexa antibodies (Molecular Probes). For examination a Zeiss LSM-500 laser scanning microscope was used.

Real-time PCR analysis

cDNA was obtained from total RNA by reverse transcription with SUPERSCRIPT™ First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. qRT-PCR was performed using the LightCycler FastStart DNA Master^{PLUS} SYBR Green I kit together with the LightCycler 2.0 System (Roche) as previously described (Wagner et al., 2006) by employing the PBGD gene as an internal loading control.

Preparation of epidermal protein lysates and Western Blot analysis

Keratinocytes were isolated as described above. Protein lysates were prepared as previously described (Sibilia et al., 2000; Wagner et al., 2006). Proteins were separated by SDS-PAGE and transferred to PVDF membranes (Millipore). Western Blot analysis was performed according to standard procedures (Sibilia et al., 2000) using the following antibodies: anti-β1 integrin (Transduction Laboratories), anti-phosphorylated

Erk1/2 (Bio Labs), anti-Erk1/2 (Santa Cruz), anti-phosphorylated Erk5 (Cell Signaling), anti-Erk5 (Cell Signaling), anti-phosphorylated Src (Bio Source), anti-Src (Bio Source), anti-phosphorylated FAK (Bio Source), anti-FAK (Neomarkers), anti-Cyclin D1 (Santa Cruz), anti-p21 (Santa Cruz), anti-Cdk1, anti-Cdk2, anti-Cdk4 and anti-p27, all from BD Transduction.

Histology and Immunohistochemistry

Mouse skin was dissected, fixed in 4% PFA and embedded in paraffin. For cryo sections skin was frozen in optimal cutting temperature compound (OCT). Sections of 5µm thickness were cut and used for histochemistry or immunofluorescence stainings. Hematoxylin and Eosin staining was carried out following standard procedures (Natarajan et al., 2007). For immunohistochemistry cryo sections were fixed in 4% PFA for 20 minutes, permeabilized with 0,1% Triton X-100/0,1% sodium citrate for 5 minutes on ice and blocked with 3% BSA/5% goat serum/PBS. The primary and secondary antibodies were diluted in 1% BSA/PBS. The following antibodies were used: anti-β1 integrin (Chemicon), anti-K1 (Covance), anti-K14 (Covance), and secondary Alexa antibodies (Molecular Probes).

Statistical methods

All experiments were repeated at least twice and done in triplicates. Data were evaluated using a Student's two-tailed t test. $p<0.05$ was taken to be statistically significant.

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FIGURE LEGENDS

Figure 1. K5-SOS expressing primary keratinocytes show increased protein levels of β1 integrin and Src kinase

(A) Keratinocytes of the indicated genotypes were used as continuously growing bulk cultures (b), or were serum- and growth factor-starved for 48 hr and stimulated with 20ng/mL EGF for 5 min. Immunoblotting was performed with the indicated antibodies, and actin was used as a loading control. **(B)** FACS analysis of skin samples with antibodies detecting cell surface β1 integrin. GD25 cells lacking β1 integrin were used as a negative control.

Figure 2. K5-SOS expression leads to increased migration of primary keratinocytes after wounding

(A-H) Phase contrast photographs of the same microscopic field of wounded keratinocytes were taken at 0h and 36h time-points after wounding. Black lines indicate wound margins **(I-L).** The Src family kinase inhibitor SU6656 was added to the medium at the indicated concentration. **(M)** Migration of wounded keratinocytes of the indicated genotypes in the presence or absence of SU6656. Data represent mean ± SEM of the number of cells which have migrated beyond the wound margins in 5-10 different fields of 3 independent experiments. * $p \leq 0.05$; ** $p \leq 0.005$; *** $p \leq 0.0005$.

Figure 3. K5-SOS expressing keratinocytes display reduced number of focal contacts after wounding

(A-H) Confocal photographs showing immunofluorescence staining of phalloidin binding to F-actin (red) and paxillin (green) in the absence **(A-D)** or presence of SU6656 **(E-H)** 36h after wounding. **(I-P)** Immunofluorescence staining of β1 integrin (red) and paxillin (green) in the absence **(I-L)** or presence of SU6656 **(M-P)**. β1 integrin

co-localizes with paxillin at sites of focal adhesion. Focal contacts are depicted by white arrows.

Figure 4. K5-SOS transgenic primary keratinocytes show constitutive activation of Erk1/2, but not of Ras

(A) Cell lysates of SU6656-treated and untreated primary keratinocytes were subjected to immunoblotting analysis with antibodies against phospho-Erk1/2, Erk1/2 and tubulin. **(B)** Keratinocytes of the indicated genotypes were serum- and growth factor-starved for 48 hr and stimulated with 20ng/mL EGF for 5 min, and subjected to immunoblotting with antibodies against phospho-Erk5, total Erk5 and actin. **(C)** Aliquots of the indicated cell lysates were subjected to pull-down with a GST fusion protein of the Ras binding domain of Raf (GST-RBD). Precipitated Ras protein (Ras-GTP) was visualized by immunoblotting with anti-Ras antibodies (upper panel). Total Ras levels in the cell lysates are shown in the lower panel. **(D)** Analysis of the indicated cell cycle proteins by Western blotting. Keratinocytes of the indicated genotypes were used as continuously growing bulk cultures (b) or were serum- and growth factor-starved for 48 hr and stimulated with 20ng/mL EGF for 5 min. Actin was used as loading control.

Figure 5. Lack of β1 integrin in the epidermis and tumors affects K5-SOS-dependent tumor development

(A,B) Phenotype of a 2 weeks-old $EGFR^{wa2/+}$ $\beta1int^{\Delta ep}$ K5-SOS mouse (black arrow) compared with a $EGFR^{wa2/+}$ $\beta1int^{\Delta ep}$ **(A)** or a $EGFR^{wa2/+}$ K5-SOS **(B)** mouse. **(C)** Tails of 2 weeks-old $EGFR^{wa2/+}$ $\beta1int^{\Delta ep}$, $EGFR^{wa2/+}$ $\beta1int^{\Delta ep}$ K5-SOS (black arrow), and $EGFR^{wa2/+}$ K5-SOS (white arrow) transgenic mice. **(D)** Tails of 3 weeks-old $EGFR^{wa2/+}$ $\beta1int^{\Delta ep}$ K5-SOS (black arrow) and $EGFR^{wa2/+}$ K5-SOS (white arrow) transgenic mice showing absence of tumors in K5-SOS transgenic mice lacking β1 integrin. **(E)** Body weight of $EGFR^{wa2/+}$ $\beta1int^{\Delta ep}$ K5-SOS, $EGFR^{wa2/+}$ $\beta1int^{\Delta ep}$, $EGFR^{wa2/+}$, and $EGFR^{wa2/+}$

K5-SOS was determined during the indicated time. **(F)** EGFR$^{wa2/+}$ β1int^{Δep} K5-SOS mice display no papilloma formation, whereas EGFR$^{wa2/+}$ K5-SOS mice develop papillomas at 100% penetrance. **(G)** EGFR$^{wa2/+}$ ß1int$^{f/f}$ K5-CreER^{T} K5-SOS mice (n=3) and EGFR$^{wa2/+}$ K5-SOS β1int$^{f/f}$ control mice (n=4) were treated intraperitoneally with tamoxifen and tumor development was measured every 2-3 days. Data represent mean ± SEM. *** p≤0.0005.

Figure 6. Epidermis of β1int^{Δep} K5-SOS mice is thickened and hyperproliferative and shows perturbed expression of keratins 1 and 14

Skin or tumors biopsies of EGFR$^{wa2/+}$, EGFR$^{wa2/+}$ K5-SOS, EGFR$^{wa2/+}$ β1int^{Δep}, and EGFR$^{wa2/+}$ β1int^{Δep} K5-SOS mice were either stained with Hematoxylin and Eosin **(A-D)** or stained with antibodies against keratin 14 **(E-H, M-P)**, keratin 1 **(I-L, M-P)** and β1 integrin **(Q-T)**. Arrows in A, B and D indicate the epidermis.

Figure 7. Molecular analysis of K5-SOS transgenic epidermis lacking β1 integrin

(A) Western blot analysis of protein lysates prepared from the epidermis of mice with the indicated genotypes. Tubulin was used as a loading control. **(B)** qRT-PCR for ß1 integrin mRNA expression in the epidermis of mice with the indicated genotypes. Data indicate the relative expression of β1 integrin mRNA normalized to expression levels of EGFR$^{wa2/+}$ epidermis. PBGD was used as an internal loading control. **(C)** Western blot analysis of protein lysates prepared from the epidermis of mice with the indicated genotypes. Tubulin was used as a loading control.

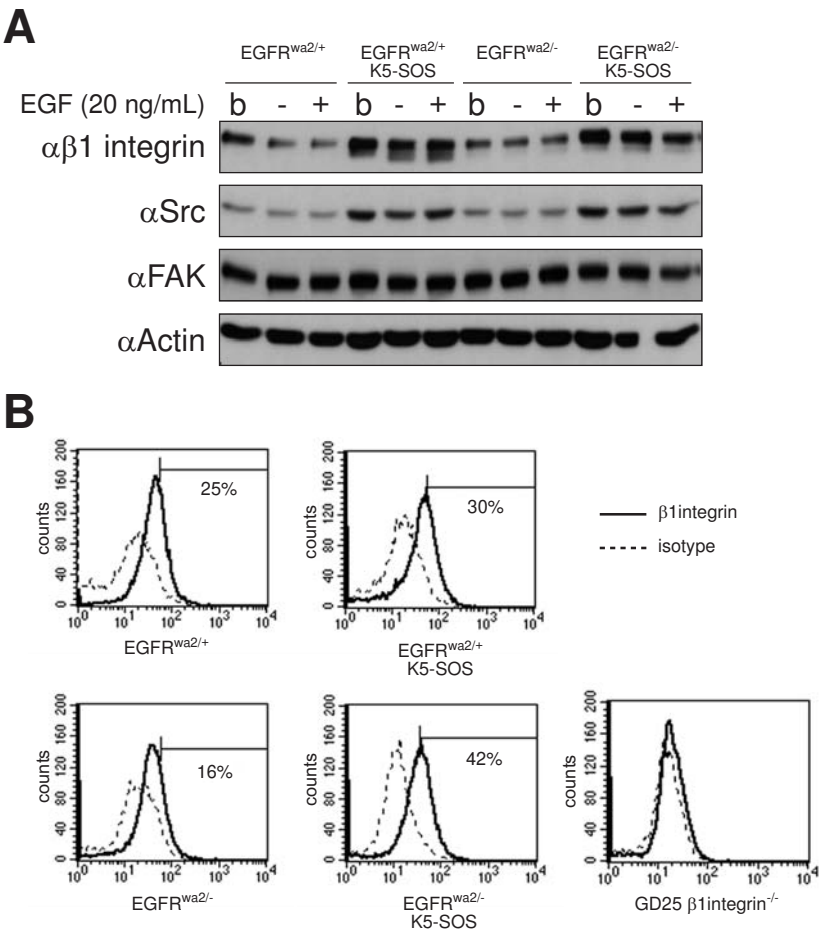
Supplementary figure 1. The phosphorylation of the regulatory tyrosine sites of Src kinase and FAK is independent of EGFR signaling

Cell lysates of primary keratinocytes stimulated for 5 min with the indicated concentrations of EGF were subjected to immunoblotting analysis with phosphorylation

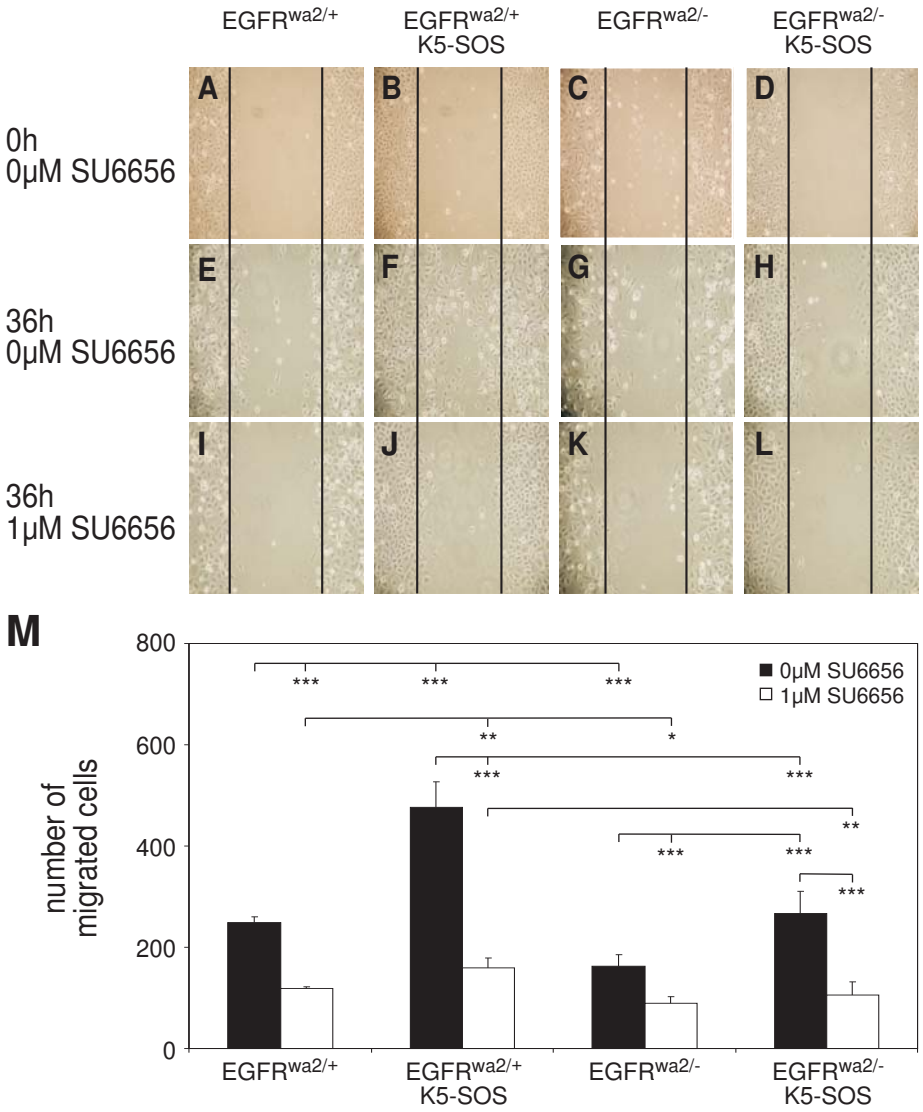
site-specific antibodies against pSrc [pY418], pSrc [pY529], and pFAK [pY397]. The identity of the Src and FAK bands and the amounts of these proteins in the cell lysates were evaluated by subsequent stripping and reprobing the blots with anti-Src and anti-FAK antibodies. Actin was used as a loading control.

Supplementary figure 2. K5-SOS expression rescues the adhesion defect of EGFR$^{wa2/-}$ keratinocytes, but does not affect the adhesion capacity of EGFR$^{wa2/+}$ keratinocytes

Primary keratinocytes of the indicated genotypes were trypsinized, treated with soy bean trypsin inhibitor, washed and subsequently resuspended in serum free medium. Single cell suspensions (10^5 cells) were incubated for 1 hr at 37°C in collagen I, collagen IV, fibronectin, vitronectin, and laminin coated wells of a Chemicon CytoMatrix™ screen kit. Adherent cells were stained with crystal violet and the absorbances at 560 nm were determined. Data represent mean ± SEM of 3 independent samples. * p≤0.05; ** p≤0.005; *** p≤0.0005.



Lichtenberger et al., Fig. 1



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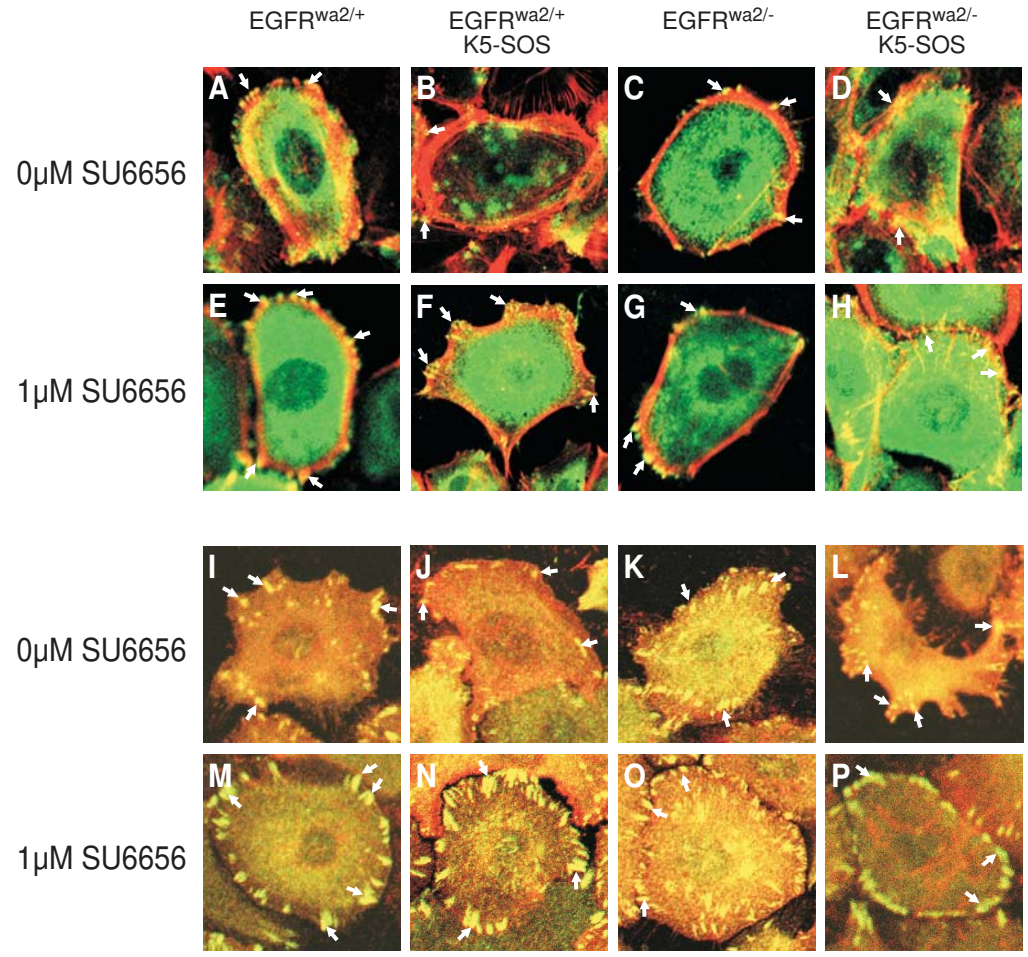

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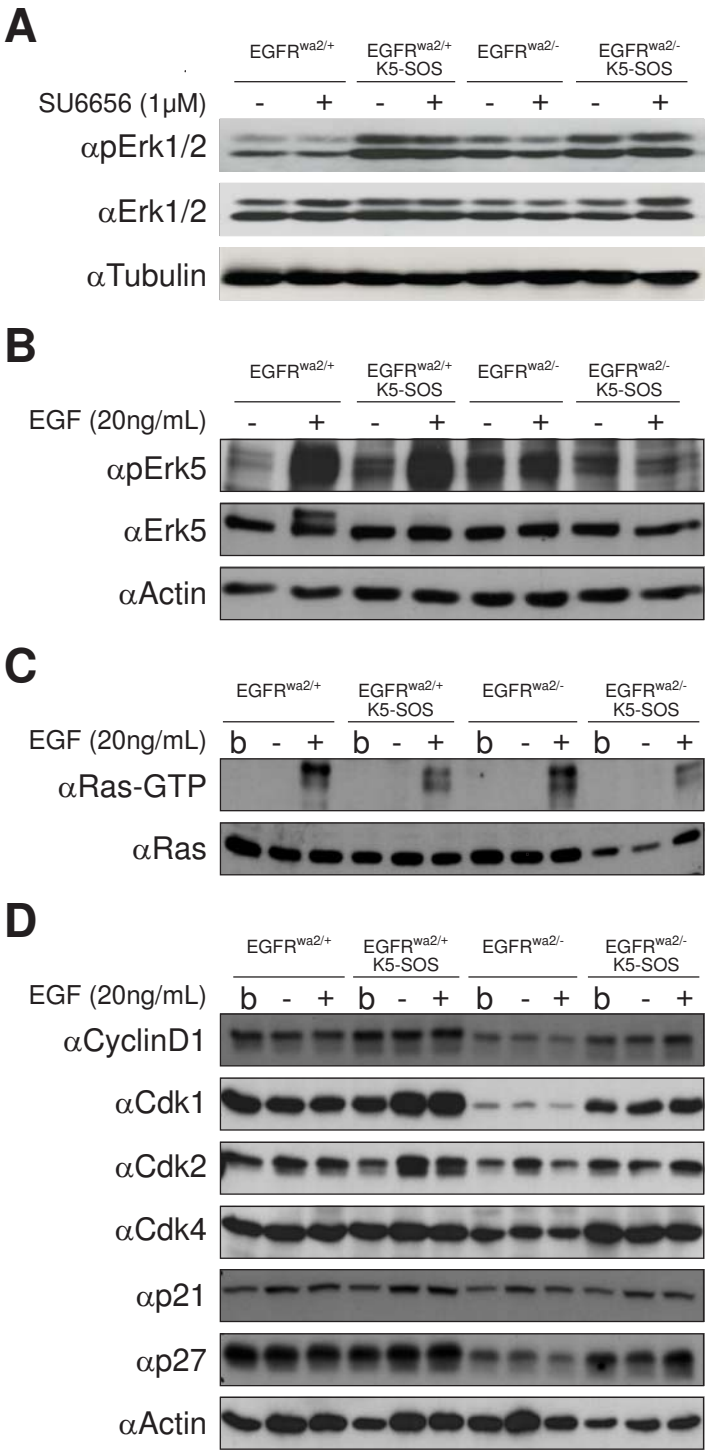

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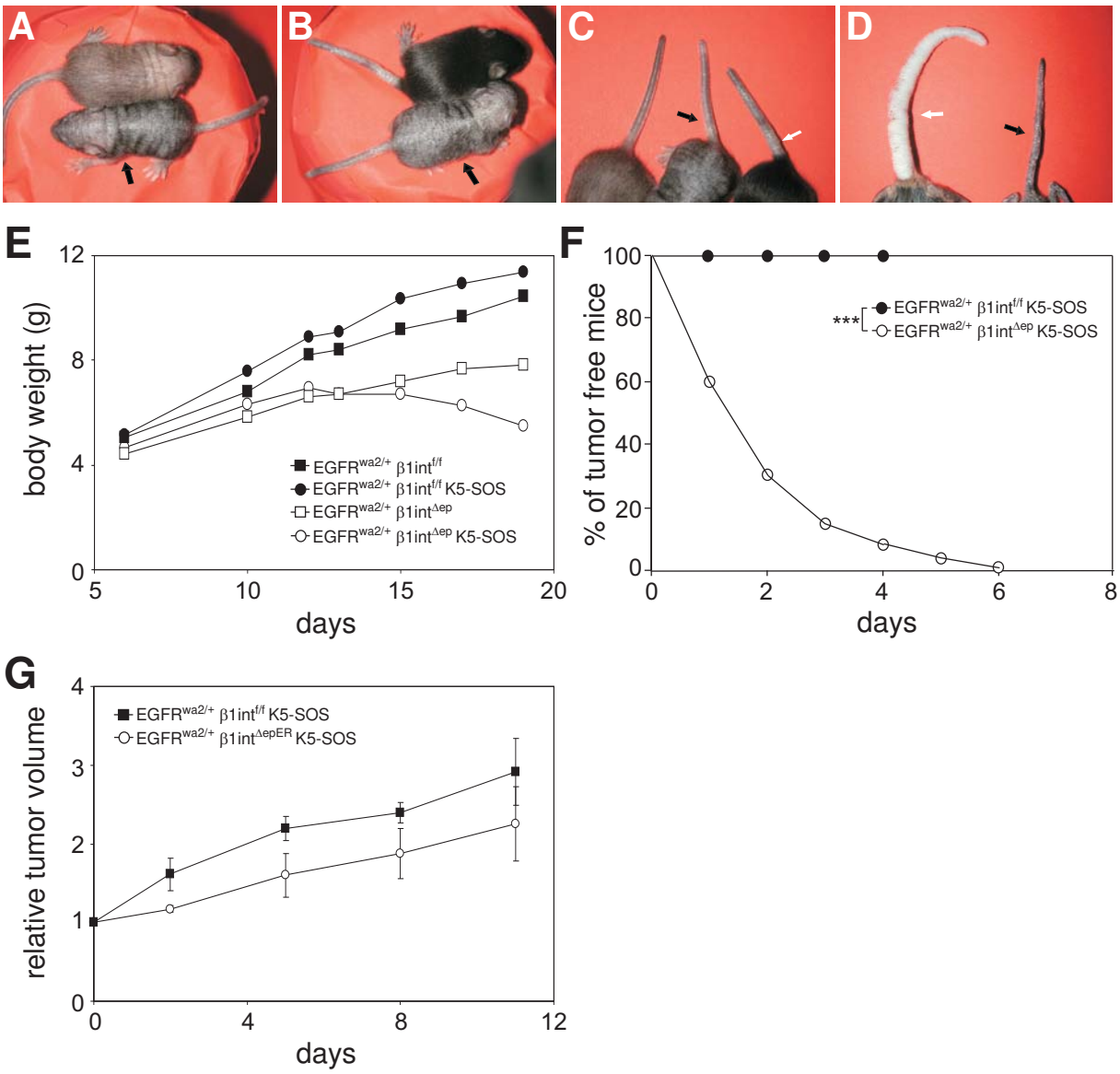

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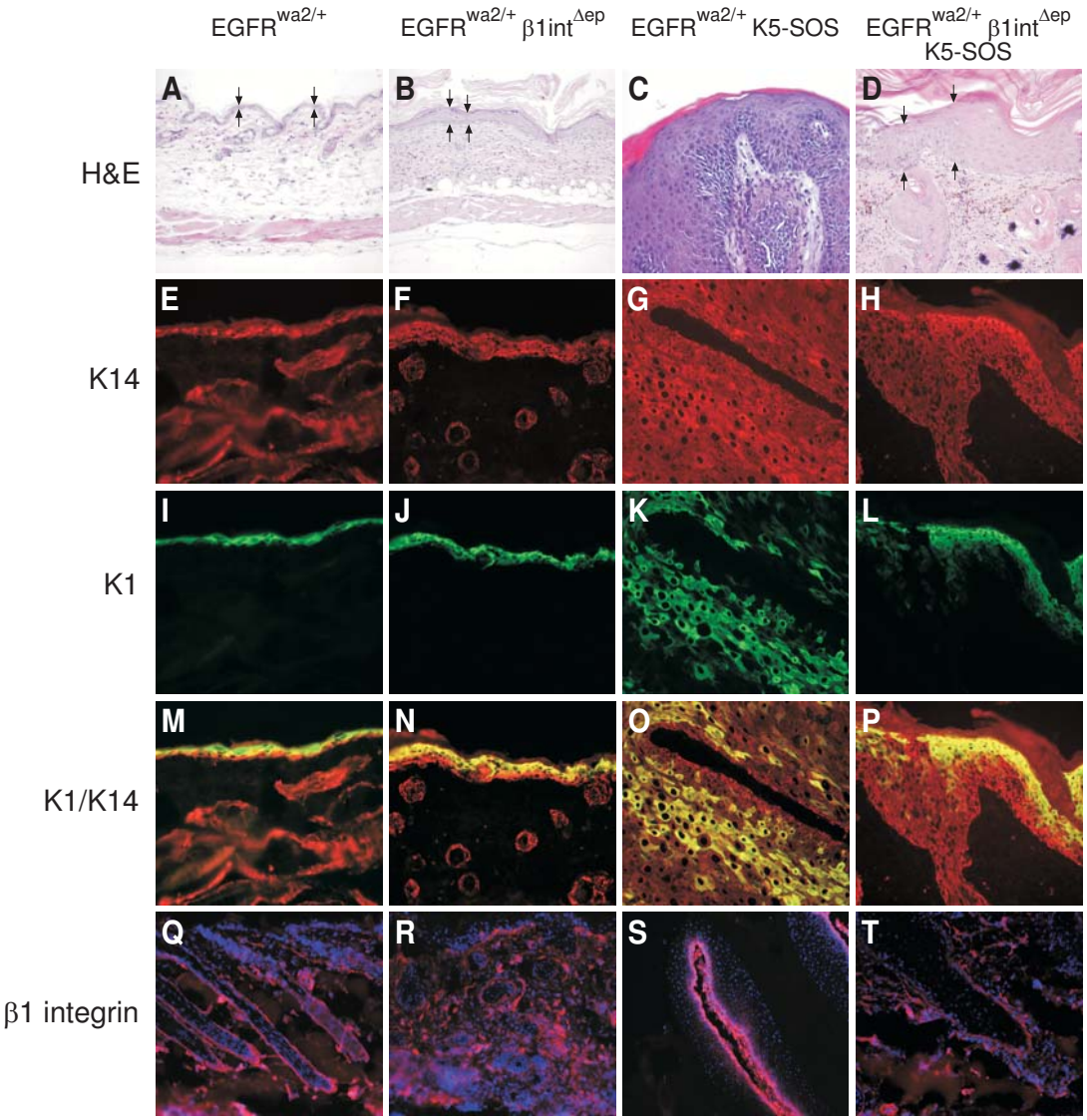

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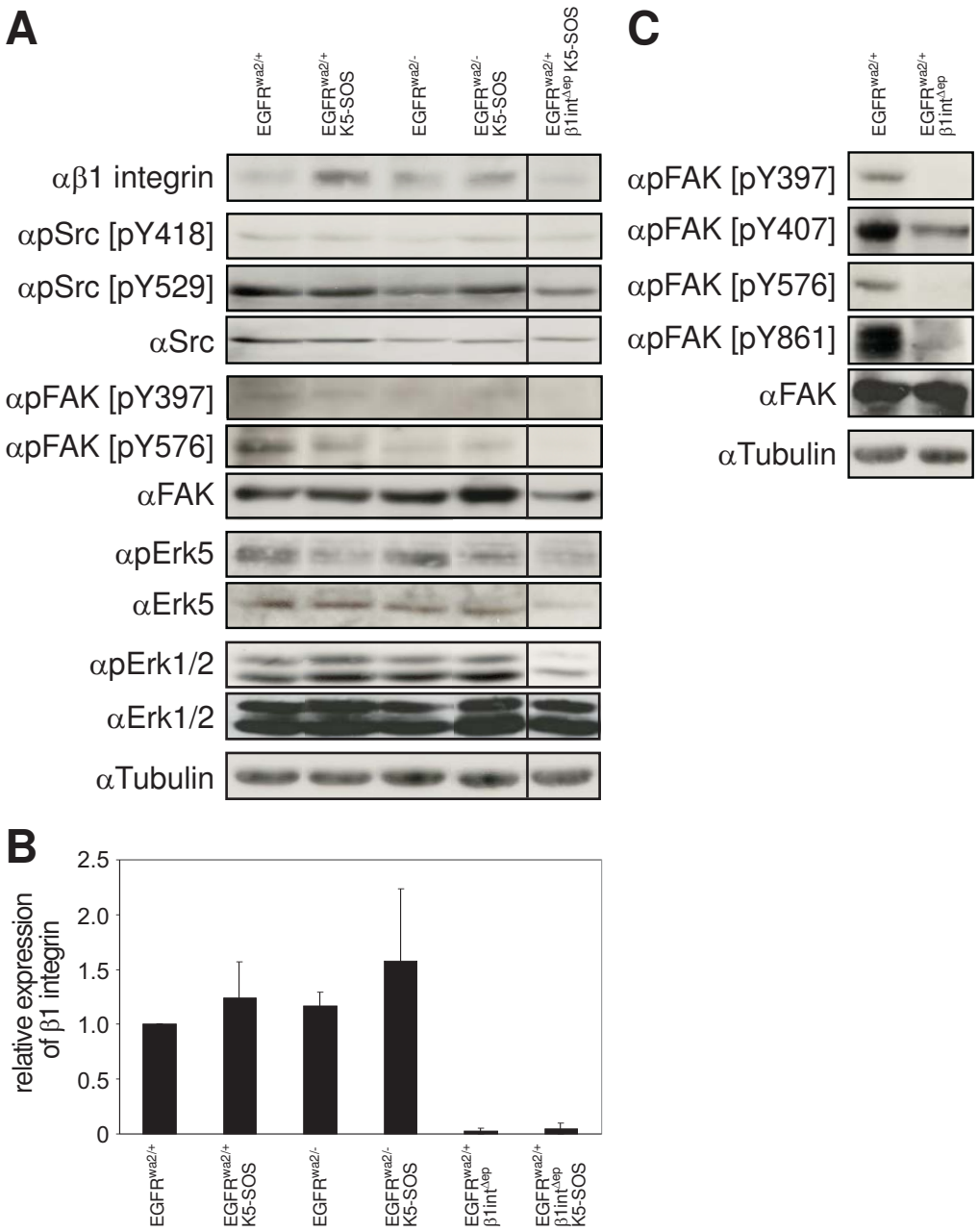

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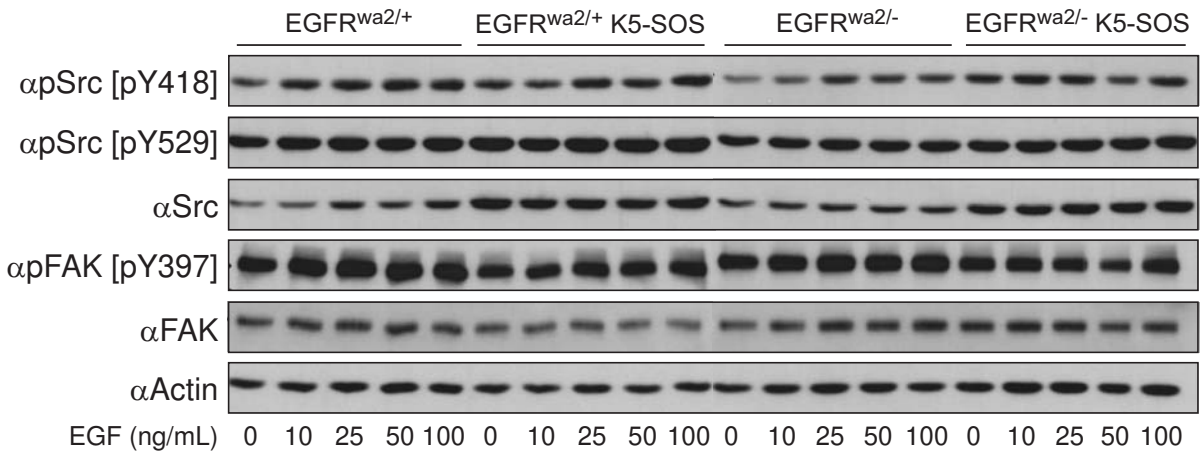

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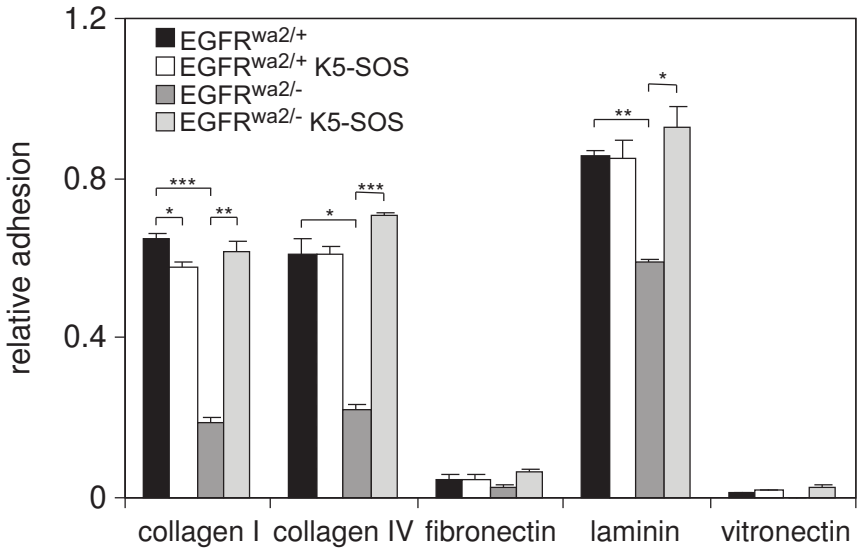

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5.4 PUBLISHED REVIEW: The epidermal growth factor receptor: from development to tumorigenesis

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REVIEW

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The epidermal growth factor receptor: from development to tumorigenesis

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Abstract The epidermal growth factor receptor (EGFR) is activated by many ligands and belongs to a family of tyrosine kinase receptors, including ErbB2, ErbB3, and ErbB4. These receptors are de-regulated in many human tumors, and EGFR amplification, overexpression, and mutations are detected at a high frequency in carcinomas and glioblastomas, which are tumors of epithelial and glial origin, respectively. From the analysis of EGFR-deficient mice, it seems that the cell types mostly affected by the absence of EGFR are epithelial and glial cells, the same cell types where the EGFR is found to be overexpressed in human tumors. Therefore, it is important to define molecularly the function of EGFR signaling in the development of these cell types, because this knowledge will be of fundamental importance to understand how aberrant EGFR signaling can lead to tumor formation and progression. A molecular understanding of the pathways that control the development of a given tissue or cell type will also provide the basis for developing better combination therapies targeting different key components of the EGFR signaling network in the respective cancerous cells. Here, we will review the current knowledge, mostly derived from the analysis of genetically modified mice and cells, about the function of the EGFR in specific organs and tissues and in sites where the EGFR is found to be overexpressed in human tumors.

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EGFR family members and signaling

The EGFR family consists of four transmembrane receptors belonging to the receptor tyrosine kinase (RTK) super family and includes EGFR (also known as ErbB1/HER-1), ErbB2/Neu/HER-2, ErbB3/HER-3, and ErbB4/HER-4 (Schlessinger, 2002). The known ligands for EGFR include epidermal growth factor (EGF), transforming growth factor-α (TGF-α), amphiregulin (AR), epiregulin (EREG), β-cellulin (BTC), and heparin-binding EGF (HB-EGF). Ligand binding to ErbB receptors induces the formation of receptor homo- and heterodimers and the activation of the intrinsic kinase domain, resulting in phosphorylation of specific tyrosine residues within the cytoplasmic tail (Yarden and Sliwkowski, 2001; Schlessinger, 2002). Phosphorylated tyrosine residues act as binding sites for proteins containing Src-homology 2 domains (SH2) such as Grb2, SHC, and PLCγ, which in turn activate complex downstream signaling cascades, thus transducing extracellular stimuli to the nucleus (Yarden and Sliwkowski, 2001; Schlessinger, 2002; Hynes and Lane, 2005). The specificity of the cellular responses is thought to be determined by the nature of the various signaling molecules recruited to the phosphorylated receptor. Together with the capacity of ErbB receptors to form homo- and heterodimers, this increases the number of signaling pathways that can be activated. Among the main pathways activated downstream of ErbB receptors are the Ras-Raf-MEK-ERK1/2, STAT3, and STAT5 pathways controlling mainly proliferation and differentiation and the PI3K-Akt-mTOR cascade acting as a

pro-survival and anti-apoptotic pathway (Yarden and Sliwkowski, 2001; Schlessinger, 2002; Hynes and Lane, 2005).

The EGFR and its family members play a pivotal role in tumor development and their expression strongly affects the clinical outcome of cancer patients (Yarden, 2001b; Hynes and Lane, 2005). EGFR overexpression in human cancer reaches 100% in tumors of the head and neck, followed by pancreatic and renal cell carcinomas, colorectal, breast, ovarian, prostate, bladder, non-small-cell lung cancer, and glioblastomas (Salomon et al., 1995; Nicholson et al., 2001; Sharma et al., 2007). In contrast, ErbB2 expression is more restricted, with approximately 30% of human breast carcinomas expressing this receptor (Hynes and Lane, 2005). In breast cancer, ErbB2 expression is associated with a poorer prognosis as compared with overexpression of EGFR alone (Yarden, 2001a; Hynes and Lane, 2005). Currently, two types of EGFR-family inhibitors are in clinical or pre-clinical use: small molecule tyrosine kinase inhibitors (TKIs) like Gefitinib (Iressa), Erlotinib (Tarceva), or Lapatinib, and antibodies such as Trastuzumab (Herceptin) and Cetuximab (Erbitux) directed against ErbB2 and EGFR, respectively. As this is not the major topic of our review, we refer to the following references for further reading (Gschwind et al., 2004; Hynes and Lane, 2005; Sharma et al., 2007).

Besides the information on EGFR expression in human tumors, the physiological function of the EGFR during organ development and function has been unclear for many years. Several attempts to overexpress the EGFR in transgenic mice, in particular from broadly expressed promoters, were unsuccessful, suggesting that increased EGFR signaling might lead to lethality during development (U. Burkert and E. F. Wagner, personal communication). The most important results on the *in vivo* functions of the EGFR were certainly obtained from loss-of-function studies, which will be described below (Fig. 1).

Phenotype of ErbB knock-out mice

Several groups generated mice lacking different members of the ErbB receptors and their ligands (Table 1). Mice lacking ErbB2 (Lee et al., 1995), ErbB3 (Riethmacher et al., 1997), ErbB4 (Gassmann et al., 1995), or the ErbB3/4 ligand heregulin (Meyer and Birchmeier, 1995) are embryonic lethal and display heart defects and abnormal development of the nervous system. In ErbB2$^{-/-}$ fetuses, the development of cranial neural crest-derived sensory ganglia is markedly affected and the development of motor nerves is also compromised. In contrast, ErbB4 knock-out mice display striking alterations in the innervations of the hindbrain and the central nervous system (CNS). Mice lacking ErbB2 or ErbB3 almost completely lack Schwann cells, which are cells of glial origin that wrap the axons of postmitotic neurons in the peripheral nervous system (Riethmacher et al., 1997; Britsch et al., 1998; Lin et al., 2000). As a consequence, motor and sensory neurons undergo cell death in later stages of development. Expression of erbB2 and erbB4 as transgenes specifically in the myocardium of erbB2- and erbB4-deficient mice, respectively, rescues the heart defects and prolongs their lifespan. Rescued erbB2-deficient mice display peripheral nervous system defects and completely lack Schwann cells, whereas rescued erbB4 mutant mice show defects in mammary gland development and display aberrant cranial nerve architecture (Woldeyesus et al., 1999; Tidcombe et al., 2003). Therefore, ErbB2, ErbB3, and ErbB4 seem to work in a cell-autonomous way during Schwann cell development and the degeneration of neurons can be attributed to the lack of factors secreted from Schwann cells.

The analysis of EGFR mutant mice revealed a complex role for this receptor during embryonic and postnatal development (Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995). Mutant mice are growth retarded and die at different stages of development depending on their genetic background. In a 129/Sv background, EGFR mutant embryos die at day 11.5 of gestation, whereas in other backgrounds mutant mice can survive until birth (C57BL/6) or to postnatal day 20 (MF1, C3H). Death *in utero* likely results from a placental defect, whereas at birth probably from lung immaturity. All surviving mutant mice show abnormalities in the bone, brain, heart, and various epithelia such as the skin, hair follicles, and eyes (Fig. 1). (Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995; Kornblum et al., 1998; Sibilia et al., 1998, 2003; Wang et al., 2004).

With the intention of studying the functional homologies between the mouse and human EGFR *in vivo*, we used a knock-in strategy to generate mice in which the endogenous mouse EGFR gene was replaced by a human EGFR cDNA (Sibilia et al., 2003). Homozygous mice humanized for the EGFR (hEGFR$^{KI/KI}$ mice) are growth retarded but can survive for up to 6 months after birth and die from heart hypertrophy. Interestingly, these mice develop tissue-specific hypomorphic phenotypes, which correlate with the expression levels of the hEGFR^{KI} allele in various tissues. For reasons that are still unclear, the expression of the hEGFR^{KI} allele is severely reduced in bone cells and epithelial tissues, whereas the hEGFR^{KI} allele is expressed at similar levels as the endogenous mouse gene in the brain. As a consequence, hEGFR$^{KI/KI}$ mice display bone, skin, and hair growth defects similar to surviving EGFR$^{-/-}$ mice. However, the neurodegeneration is fully rescued. Moreover, higher levels of expression of the hEGFR^{KI} allele are detected in the heart and are likely responsible for the development of the heart hypertrophy. These results demonstrate that mice humanized for EGFR display

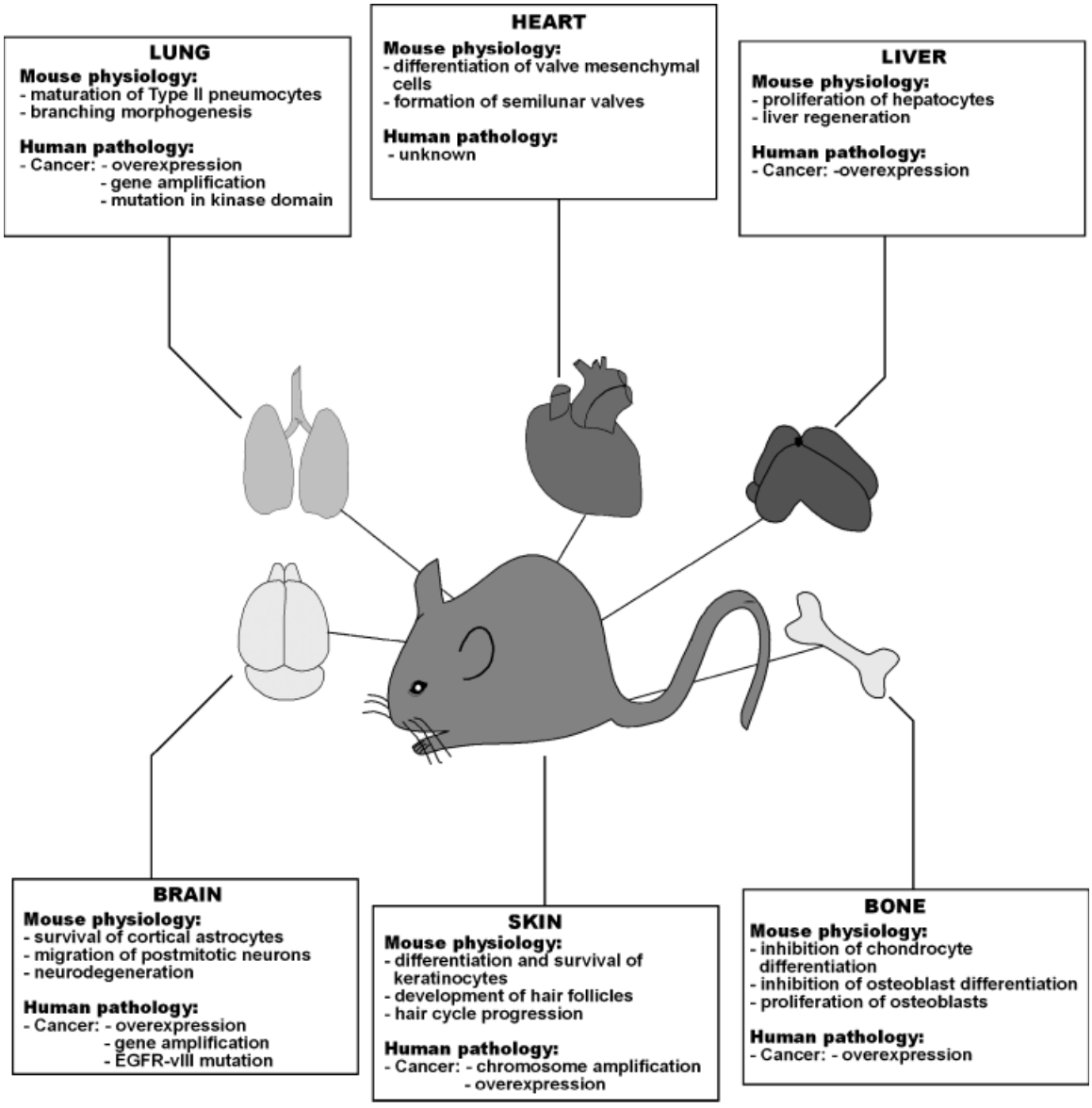

Fig. 1 Function of the EGFR during the development of different mouse organs. EGFR, epidermal growth factor receptor.

tissue-specific hypomorphic phenotypes, thereby uncovering novel functions of the EGFR in bone and heart development (Sibilia et al., 2003).

In contrast to the multiorgan defects observed in EGFR mutant mice, deletion, or overexpression of EGFR ligands such as TGF-α, EGF, and AR either leads to no phenotype or to very mild defects in the skin, hair, bone, and mammary gland, suggesting that there is high redundancy among the ligands in the different organs (Luetteke et al., 1993, 1999; Cook et al., 1997; Chan and Wong, 2000; Wong et al., 2000).

EGFR in placental development

EGFR mutant placentas of all genetic backgrounds exhibit a structurally comparable labyrinthine trophoblast layer but a severely reduced spongiotrophoblast layer when compared with controls (Figs. 2A–2F). In a 129/Sv EGFR mutant background, this defect most likely leads to lethality of 100% of the embryos whereas in a C57BL/6, MF1, and C3H background 40%–50% of the mutant fetuses can still survive until birth with the same placental defect. When we compared wild-type (wt) placentas from different mouse strains, we noticed that 129/Sv placentas were smaller in size and less robust than those of other backgrounds (our unpublished observations). It is therefore possible that despite the spongiotrophoblast defect, mutant placentas of C57BL/6, MF1, and C3H can still provide sufficient maternal-fetal adhesiveness and nutrition supply to allow a certain fraction of embryos to survive longer than midgestation. The 129/Sv embryonic lethality can be

Table 1 ErbB receptors and ligands: phenotype of genetically modified mice

Gene	Alteration	Phenotype	References
EGFR	Knock-out	Die between mid-gestation and postnatal day 20 depending on the genetic background, epithelial defects in skin, hair, eyes and lungs, bone and heart abnormalities, neurodegeneration, defect in cortical astrocytes	Sibilia and Wagner (1995), Sibilia et al. (1998, 2003), Miettinen et al. (1995), Threadgill et al. (1995), Kornblum et al. (1998), Wang et al. (2004), Wagner et al. (2006)
	Knock-in	Hypomorphic phenotypes of skin, bone, and heart	Sibilia et al. (2003)
	wa2, spontaneous mutation	Skin and hair abnormalities, impaired lactation	Luetteke et al. (1994), Fowler et al. (1995)
ErbB2	Knock-out	Embryonic lethal, neural, and cardiac defects, Schwann cell defects	Lee et al. (1995), Lin et al. (2000)
ErbB3	Knock-out	Embryonic lethal, lack of Schwann cell precursors, degenerated peripheral nervous system	Riethmacher et al. (1997)
ErbB4	Knock-out	Embryonic lethal, cardiac and neural defects, mammary gland defects	Gassmann et al. (1995), Tidcombe et al. (2003)
TGF-α	Knock-out	Abnormal skin architecture, hair follicle, and eye abnormalities	Mann et al. (1993), Luetteke et al. (1993)
	Overexpression	Epithelial hyperplasia, mammary gland, and pancreatic abnormalities, liver neoplasia	Sandgren et al. (1990), Jhappan et al. (1990)
	wal, spontaneous mutation	Skin and hair abnormalities	Luetteke et al. (1993)
EGF	Knock-out	No overt phenotype	Luetteke et al. (1999)
	Overexpression	Infertile, growth retardation	Chan and Wong (2000), Wong et al. (2000)
HB-EGF	Knock-out	Heart abnormalities	Iwamoto et al. (2003)
AR	Knock-out	Mild phenotype in mammary glands	Luetteke et al. (1999)
	Overexpression	Psoriasis-like phenotype	Cook et al. (1997)
NRG1	Knock-out	Embryonic lethal, heart malformation, lack of Schwann cell precursors and cranial ganglia	Meyer and Birchmeier (1995)
TGF-α EGF AR	Triple knock-out	Hair abnormalities and eye defects, impaired mammary gland function	Luetteke et al. (1999)
ErbB2 ErbB3 NRG1	Triple knock-out	Embryonic lethal, severe hypoplasia of the primary symphathetic ganglion chain	Britsch et al. (1998)

EGF, epidermal growth factor; EGFR, EGF-receptor; TGF, transforming growth factor; AR, amphiregulin; NRG, neuregulin; HB-EGF, heparin binding EGF; wal, waved-1; wa2, waved-2.

rescued by generating aggregation chimeras between EGFR mutant and tetraploid wt embryos, the latter contributing exclusively to the extraembryonic tissues. When provided with a wt placenta, EGFR mutant mice of pure 129/Sv genetic background survive up to postnatal day 20 and show defects similar to those of mutants of other backgrounds (Sibilia et al., 1998). These results confirm that the placental defects are responsible for the embryonic lethality of 129/Sv EGFR mutant embryos. The viability of EGFR mutant newborns can be improved by inducing lung maturation by transplacental administration of dexamethasone, a hormone known to promote lung maturation, demonstrating that lung immaturity is responsible for the high perinatal mortality (Sibilia et al., 1998). All tetraploid rescued 129/Sv and dexamethasone-treated EGFR mutant mice surviving up to 20 days after birth are severely growth retarded and develop the same defects as spontaneously surviving mice of MF1 and C3H backgrounds (Sibilia et al., 1998). This demonstrates that the postnatal phenotypes including the growth retardation occurs independently from the placental defects and that the genetic background most likely influences the development of extraembryonic tissues.

EGFR in heart development

A severe heart phenotype is observed in mice humanized for the EGFR. $hEGFR^{KI/KI}$ mice can survive up to 6 months after birth and develop a severe heart hypertrophy with dramatically increased thickness of the left ventricular wall and the interventricular septum, which becomes apparent 3 weeks after birth and progresses with age (Sibilia et al., 2003). Interestingly, $EGFR^{-/-}$ mice did not display signs of hypertrophy at the age of 3 weeks, suggesting that this phenotype was not due to the lack of EGFR expression. As heart-specific expression of the $hEGFR^{KI}$ allele seemed to be higher than the endogenous wt allele, it is likely that increased EGFR signaling in cardiomyocytes is contributing to the development of the heart hypertrophy.

$hEGFR^{KI/KI}$ mice also display semilunar valve defects, which are known to induce aortic stenosis and regurgitation, and as a consequence can lead to heart

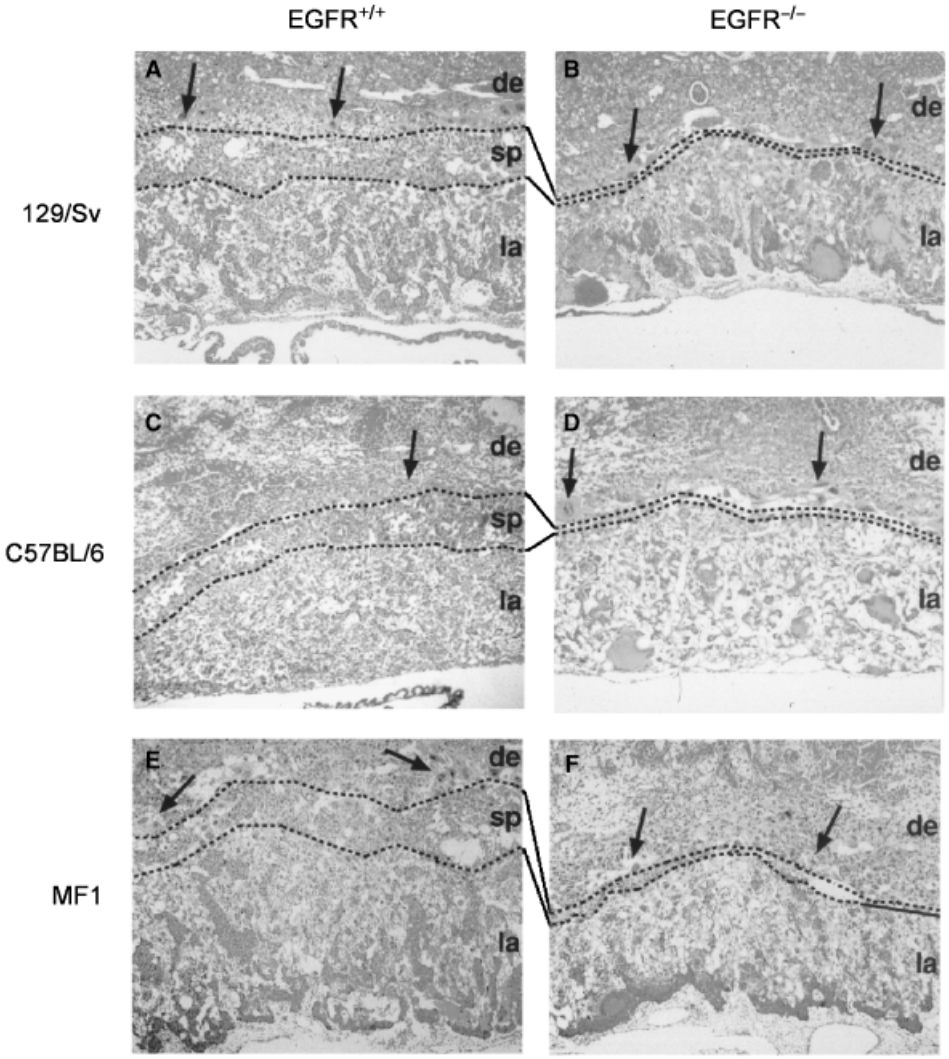

Fig. 2 Histological sections of placentas isolated at E11.5 from control (**A**, **C**, **E**) and EGFR$^{-/-}$ embryos (**B**, **D**, **F**) of 129/Sv (A, B), C57BL/6 (C, D), and MF1 (E, F) genetic backgrounds. Note that in mutant placentas of all three genetic backgrounds, the spongiotrophoblast layer (sp, delimited with dotted lines) that is localized between the maternal decidua (de)/embryonic giant cells (arrows) and the labyrinthine trophoblast layer (la) is severely reduced in size. The labyrinthine trophoblast layer appears to be structurally similar in all backgrounds and is comparable to the controls. EGFR, epidermal growth factor receptor.

hypertrophy. The pulmonary and aortic, but not the atrioventricular valves of hEGFR$^{KI/KI}$ hearts were thickened and hypercellular (Sibilia et al., 2003). Because the same phenotype was also observed in EGFR$^{-/-}$ mice as well as in the naturally occurring mouse mutant strain waved-2 (wa2), which carries a point mutation in the EGFR gene leading to reduced kinase activity, it is likely that the hEGFR^{KI} allele is not expressed in the developing valves (Luetteke et al., 1994; Fowler et al., 1995; Sibilia et al., 2003). Similar cardiac valve phenotypes are present in mice lacking HB-EGF, a ligand for EGFR and ErbB4, suggesting that EGFR/HB-EGF signaling is required for the differentiation of valve mesenchymal cells within the valve leaflets (Fig. 1) (Iwamoto et al., 2003; Sibilia et al., 2003).

As there are no signs of myocardial hypertrophy in 3-week-old EGFR$^{-/-}$ mice, it is likely that the severe hypertrophy observed in hEGFR$^{KI/KI}$ mice results from the malformations of the valves and from the enhanced hypertrophic response of cardiomyocytes to increased EGFR signaling, as the hEGFR^{KI} allele is expressed at higher levels in the myocardium (Sibilia et al., 2003). These defects can lead to a severe heart condition, which is probably responsible for the lethality of hEGFR$^{KI/KI}$ mice, whereas the valve defects alone, as seen in EGFR$^{wa2/wa2}$ mice, do not seem to increase the mortality of these mice.

EGFR in brain development and tumors

All EGFR family members are involved in neural development as demonstrated by neural defects upon targeted gene deletions (Table 1). Mice lacking the EGFR

develop a progressive neurodegeneration starting after birth in the frontal cortex and olfactory bulb and thereafter extending to the thalamus, leading to wide-ranging neuronal loss in the brain (Kornblum et al., 1998; Sibilia et al., 1998). This degeneration is characterized by massive apoptotic cell death that affects neurons and glial cells, which comprise astrocytes and oligodendrocytes in the CNS. In addition, a migratory disorder is detected in the hippocampus with nests of ectopic neurons, which are also undergoing apoptosis. Because the EGFR is expressed in the affected cell types, these results suggest that the EGFR controls the proliferation and/or differentiation of astrocytes as well as the survival and migration of postmitotic neurons (Sibilia et al., 1998).

In cerebral cortices of $EGFR^{-/-}$ mice, lower astrocyte numbers are observed and their expansion *in vitro* is severely compromised (Kornblum et al., 1998; Sibilia et al., 1998). Interestingly, a vigorous "reactive astrogliosis" is observed in the thalamic regions of EGFR mutant mice, indicating that astrocytes are not impaired in this region (Sibilia et al., 1998). Biochemical and functional differences have been reported among astrocytes derived from anatomically distinct regions of the brain and our laboratory could recently demonstrate that in the absence of EGFR only cortical astrocytes are affected (Wagner et al., 2006). EGFR signaling seems to play a key role in controlling cortical neurodegeneration by regulating cortical astrocyte apoptosis, thereby providing a mechanism for the region-specific neurodegeneration in $EGFR^{-/-}$ mice. Whereas $EGFR^{-/-}$ midbrain astrocytes are unaffected, mutant cortical astrocytes display increased apoptosis mediated by an Akt-caspase-dependent mechanism. As a consequence, cortical $EGFR^{-/-}$ astrocytes are unable to support neuronal survival while midbrain astrocytes are competent to keep neurons alive independently of EGFR expression (Wagner et al., 2006). These results suggest that neuronal loss occurs as a consequence of increased astrocyte apoptosis. Interestingly, neuron-specific expression of activated Ras can compensate for the deficiency of $EGFR^{-/-}$ cortical astrocytes and prevent neuronal death. These results identify two functionally distinct astrocyte populations, which differentially depend on EGFR signaling for their survival and also for their ability to support neuronal survival (Wagner et al., 2006). Whether regional variations in glial function influence the pathology of different human neurodegenerative diseases is a fascinating hypothesis to be tested in future.

The findings about the astrocyte defects are intriguing in view of the fact that EGFR amplification or activating mutations are observed at a very high frequency in glioblastoma multiforme (GBM) patients. Glioblastomas are among the most frequent brain tumors derived from glial cells and despite neurosurgery, chemo-, and radiotherapy, patient survival is very poor. Gliomas are divided into four clinical grades (WHO grade I–IV) based on histology and prognosis with grade IV known as GBM (Kleihues et al., 2002). Two different subtypes of GBM can be distinguished and are classified as primary or secondary glioblastomas. Primary glioblastomas appear to develop *de novo*, occurring in older patients, causing 95% of GBM. The majority of cases display overexpression, gene amplification, and/or activating mutations of EGFR whereas alterations in p53 are very rare. In contrast, in secondary glioblastomas, which progress from low-grade astrocytomas and occur in younger patients, mutations of p53 are very frequent whereas alterations of EGFR are rare. It seems, therefore, that p53 mutations and EGFR amplification are mutually exclusive in GBM (Watanabe et al., 1996). Hence, primary and secondary glioblastomas might originate from two different types of astrocytes and/or astrocyte progenitors: one susceptible to aberrant EGFR signaling and one not.

Several genomic rearrangements occur in human glioblastomas. In the largest population-based study, loss of heterozygosity (LOH) at 10q was identified to be most common (69%), followed by EGFR gene amplification (34%), p53 mutations (31%), $p16^{ink4a}$ deletions (31%), and PTEN mutations (24%) (Ohgaki et al., 2004). In addition to EGFR amplification, alterations in the EGFR gene structure such as deletions of parts of the extracellular domain (EGFR vI–vV) are often found in human GBM. A very frequent variant is the EGFRvIII mutation, which carries an in-frame deletion of exon 2–7 resulting in a constitutively active protein that is less sensitive to degradation and capable of phosphorylating downstream targets (Nicholas et al., 2006). Altogether, alterations in EGFR signaling are found in up to 63% of glioblastomas (Watanabe et al., 1996). The role of EGFR in gliomagenesis has been confirmed in various mouse models over the past few years, although a transgenic model recapitulating human disease is, unfortunately, still not available (Table 2).

The first mouse model was using a retroviral approach to target overexpression of the constitutively active EGFRvIII in either nestin+ neuronal precursors or GFAP+ astrocytes (Holland et al., 1998). EGFRvIII expression induces glioma-like lesions only in ink4a-arf-deficient mice, confirming that a combination of genetic alterations is necessary for tumor development (Table 2). Similarly, implantation of either nestin+ or GFAP+ cells expressing either wt or mutant EGFRvIII do not give rise to tumors in SCID mice unless ink4a/Arf is additionally deleted. In this case, both nestin+ and GFAP+ cells overexpressing mutant EGFR are able to induce high-grade gliomas at 100% penetrance (Bachoo et al., 2002). Another mouse model expressing oncogenic Ras in GFAP+ cells (GFAP-V^{12} Ha-ras) develops astrocytomas and additional expression

Table 2 Mouse models investigating the role of EGFR in tumor

Mouse model	Phenotype	References
Brain		
EGFRvIII in nestin+/GFAP+ cells	No tumors	Holland et al. (1998)
EGFRvIII in nestin+ cells; ink4a-arf$^{-/-}$	Glioma-like lesions	Holland et al. (1998)
EGFRvIII in GFAP+ cells; ink4a-arf$^{-/-}$	Glioma-like lesions	Holland et al. (1998)
GFAP- V^{12}Ha-ras; EGFRvIII	High-grade oligodendrogliomas	Ding et al. (2003)
S100β-v-erbB	Low-grade oligodendrogliomas	Weiss et al. (2003)
S100β-v-erbB; ink4a/arf$^{+/-}$	High-grade oligodendrogliomas	Weiss et al. (2003)
S100β-v-erbB; p53$^{+/-}$	High-grade oligodendrogliomas	Weiss et al. (2003)
CNP-EGFR	Enlarged nerves	Ling et al. (2005)
CNP-EGFR; NF1$^{+/-}$	Enlarged nerves, rare nerve tumors	Ling et al. (2005)
NF1$^{+/-}$; p53$^{+/-}$; EGFR$^{wa2/wa2}$	Less tumors compared with NF1$^{+/-}$; p53$^{+/-}$	Ling et al. (2005)
Skin		
K5-dnEGFR (CD533)	Skin and hair defects	Murillas et al. (1995)
K5-dnEGFR, Ras	Smaller skin papillomas, reduced vascularization	Casanova et al. (2002)
K5-ErbB2	Papillomas, SCC	Kiguchi et al. (2000)
K14-TGF-α	Benign papillomas upon wounding	Vassar and Fuchs (1991)
K1-TGF-α	Spontaneous and inducible tumors	Dominey et al. (1993), Wang et al. (1994)
K5-SOS-F	Papillomas 100% penetrance	Sibilia et al. (2000)
K5-SOS-F; EGFR$^{-/-}$/EGFR$^{wa2/wa2}$	Impaired tumor development	Sibilia et al. (2000)
K5-SOS; c-jun$^{\Delta ep}$	Smaller papillomas with low EGFR expression	Zenz et al. (2003)
Mig6$^{-/-}$	Spontaneous tumors in various organs	Ferby et al. (2006)
Mig6$^{-/-}$; EGFR$^{wa2/wa2}$	Rescue	Ferby et al. (2006)
Lung		
EGFR^{L858R}, inducible	Adenocarcinomas	Politi et al. (2006)
EGFR$^{\Delta L747-S752}$, inducible	Adenocarcinomas	Politi et al. (2006)
Liver		
Metallothionein (MT)-TGF-α	Liver, pancreas, mammary gland neoplasia	Jhappan et al. (1990), Sandgren et al. (1990)
Alb-c-myc, MT-TGF-α	Accelerated liver neoplasia	Murakami et al. (1993), Sandgren et al. (1993)

GFAP, glial fibrillary acidic protein; CNP, 2′,3′-cyclic nucleotide 3′-phosphodiesterase; EGFR, epidermal growth factor receptor; TGF, transforming growth factor.

of EGFRvIII, but not wt EGFR, accelerates tumor formation (Ding et al., 2003). The resulting tumors resemble oligodendrogliomas and a minority of them is characterized as mixed oligoastrocytomas. Also in this model the overexpression of either wt or mutant EGFR alone in GFAP+ cells does not result in glioma formation and only an increase in astrocyte numbers is observed (Table 2) (Ding et al., 2003).

About 5%–18% of gliomas are characterized as oligodendrogliomas and are probably derived from oligondendrocytes based on marker expression. Similar to glioblastomas, about 50% of oligodendrogliomas are reported to express high amounts of EGFR (Reifenberger et al., 1996). Interestingly, mice expressing v-erbB, an oncogenic version of the EGFR, in oligodendrocytes from the S100β promoter develop oligodendrogliomas reflecting the pathology of the human disorder (Weiss et al., 2003). Additional alterations like loss of p53 or ink4a/arf are necessary to induce a transition from low- to high-grade tumors in these transgenic animals (Table 2). Although S100β is also expressed in astrocytes during early development, no transformation of these cells is observed indicating cell type-dependent differences in the transforming capacity of the v-erbB oncogene.

Apart from gliomas, EGFR gene amplifications are also detected in malignant peripheral nerve sheath tumors (MPNST) (Perry et al., 2002). Neurofibromatosis type 1 (NF1) patients are susceptible to MPNSTs and Schwann cells play a key role in neurofibroma formation. NF1 patients have a loss of function of neurofibromin (NF), a tumor suppressor inhibiting Ras. Schwann cells normally do not express EGFR but only ErbB2 and ErbB3. Nevertheless, aberrant expression of EGFR is found in Schwann cell-derived tumors of NF1 patients (DeClue et al., 2000). In a mouse model, overexpressing EGFR in Schwann cells (CNP-EGFR), changes in nerve size are observed, reflecting the hallmarks of human neurofibromas. However, tumor formation is very rare and heterozygosity for NF1 does not influence the result (Table 2) (Ling et al., 2005).

The different approaches discussed above to study tumorigenesis in mice recapitulate to some extent both genetic abnormalities and histologic features of human brain tumors. Overexpression of wt EGFR is not sufficient to initiate tumor formation but a second genetic

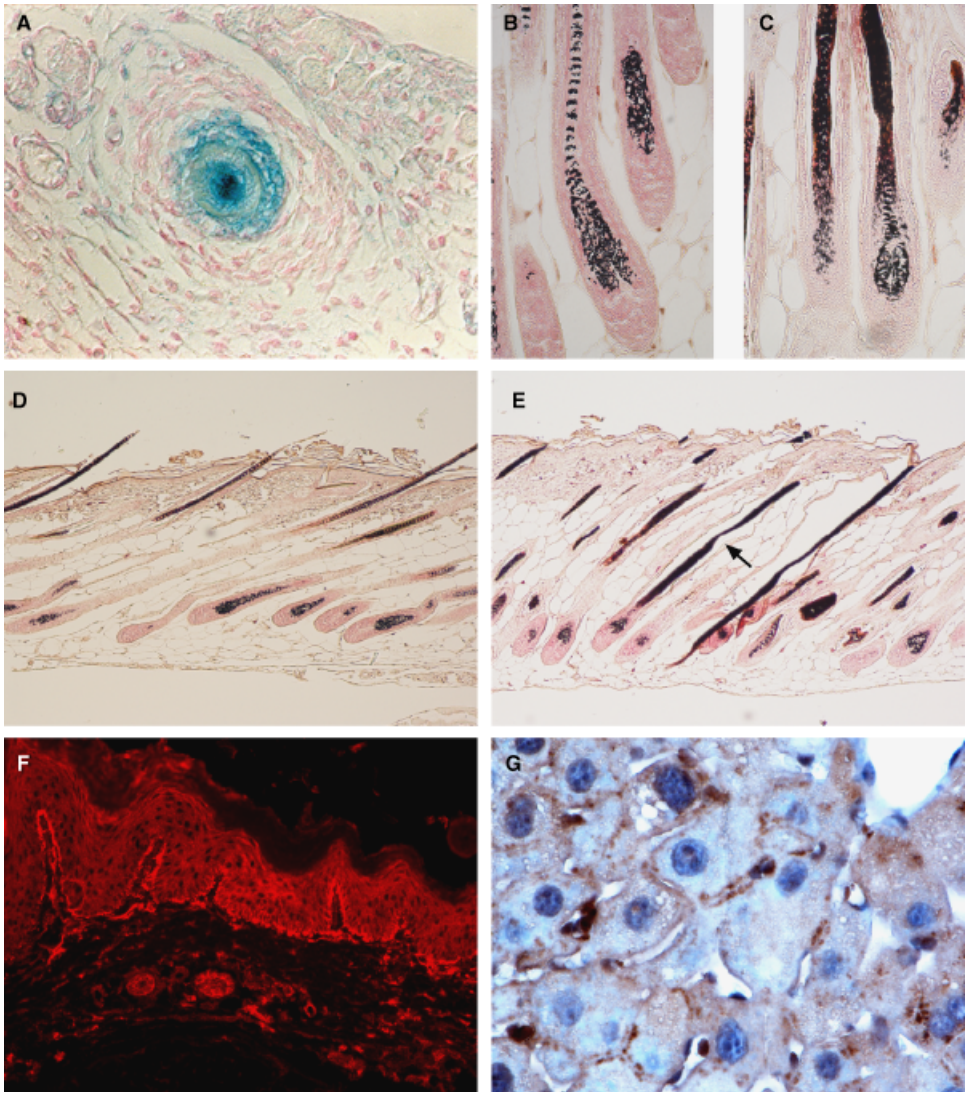

Fig. 3 (**A**) Cross-section of an HF from an EGFR$^{+/-}$ mouse showing EGFR expression (blue stain) by X-galactosidase staining. (**B–E**) Fontana–Masson staining on skin samples from an EGFR$^{-/-}$ mouse (C, E) and a littermate control (B, D) at postnatal day 10. EGFR$^{-/-}$ mice show a delay in hair follicle morphogenesis. Mutant hair follicles are irregularly and more intensively pigmented (C) than control (B). The reason for the irregular shape of EGFR$^{-/-}$ hair follicles is most probably the separation of the inner and outer root sheaths indicated by the arrow (E). (**F, G**) Immunohistological stainings showing EGFR expression in the basal cells of a K5-SOS-induced skin papilloma (F) and chemically induced hepatocellular carcinoma (G). EGFR, epidermal growth factor receptor; SOS, Son of Sevenless.

alteration such as deletion of the ink4a gene is necessary. Overexpression of EGFR mutants normally does not lead to tumor formation, except for the S100β-driven expression of v-erbB initiating the development of low-grade oligodendrogliomas. Based on these observations and also from human molecular clinical data, one can speculate that alterations in EGFR expression are rather relevant in glioma progression than initiation (Ding et al., 2003).

A longstanding debate in neuro-oncology is ongoing about the origin of the cells initiating glial tumor formation. For high-grade malignant gliomas, it seems that both astrocytes and neural stem cells can give rise to tumors, demonstrating that genetic alteration rather than the degree of cellular differentiation influences tumor development (Bachoo et al., 2002). Other studies have identified subpopulations of human brain cancer cells displaying stem-cell characteristics and being responsible for tumor growth (Singh et al., 2003, 2004). Evidence for cancer stem cells in brain tumors is provided by the p53;NF1 astrocytoma mouse model. In mice mutant for both p53 and NF1 tumors initiate in the stem cell harboring subventricular zone (SVZ) although all brain cells are genetically altered (Zhu et al., 2005). The identification of a tumor-initiating cell subpopulation expressing the neural precursor marker

CD133 proves the existence of cancer stem cells in human brain tumors (Singh et al., 2004). The presence of tumor stem cells enables additional therapeutic interventions and the combination of EGFR inhibition together with cancer stem cell elimination might help to reduce cancer recurrence and improve the survival of patients.

EGFR in skin development and tumors

EGFR has long been known to play an important role in regulating the development of the epidermis and its appendages. In the skin, EGFR is most abundantly expressed in the basal layer of the epidermis and in the outer root sheath of the hair follicles (Fig. 3A), where the proliferating cells reside. EGFR expression is downregulated as soon as keratinocytes differentiate and migrate to the suprabasal epidermal layers (Sibilia and Wagner, 1995). A number of naturally occurring and experimentally induced mutant mice confirm that EGFR signaling is of physiological relevance during normal epithelial development. Mice homozygous for a disrupted TGF-α gene have severe derangements of hair follicles, resulting in a wavy coat and curly whiskers (Table 1) (Mann et al., 1993). A similar phenotype is found in the naturally occurring mouse mutant strains wal and wa2, which carry null mutations in the TGF-α gene and hypomorphic mutations in the EGFR, respectively (Luetteke et al., 1994; Fowler et al., 1995). Similarly, EGFR-deficient mice show strain-dependent defects in epidermal as well as hair follicle differentiation (Figs. 3B–3E) and fail to develop a hairy coat, most likely because EGFR signaling is essential for maintenance of hair follicle integrity (Luetteke et al., 1994; Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995; Hansen et al., 1997). Because $EGFR^{-/-}$ mice do not survive longer than 3 weeks, hair follicle development and cycling could not be carefully analyzed. Mice humanized for the EGFR proved to be extremely useful for this purpose and revealed that after the first hair cycle, EGFR-deficient hair follicles fail to enter into catagen and remain in a berrant anagen. With time, the follicles are degraded, leading to massive infiltration of inflammatory cells, and $hEGFR^{KI/KI}$ mice are completely bald by the age of 6 months (Sibilia et al., 2003). Similar skin and hair defects are observed in transgenic mice expressing a dominant-negative human EGFR (CD533) in the basal layers of the epidermis (Murillas et al., 1995). These findings reveal that EGFR signaling is needed to regulate hair cycle progression and to preserve hair follicle integrity by controlling the proliferation, differentiation, and survival of epithelial cells.

Skin cancer is the third most common human malignancy, and its incidence has been increasing at an alarming rate over the past decades, with basal cell carcinoma (BCC), squamous cell carcinoma (SCC), and melanoma being the most common forms. Each year, an estimated number of 2–3 million non-melanoma skin cancer and 132,000 cases of melanoma occur (World Health Organization). In human SCCs, amplification of the EGFR is very common (Nicholson et al., 2001; Maubec et al., 2005). On the contrary, focal amplification and/or mutation of EGFR have not been reported in melanomas. However, late-stage melanomas often display EGFR overexpression in association with increased copies of chromosome 7 on which the human EGFR gene is localized (Chin et al., 2006).

Several studies in mouse models demonstrate that alterations in the EGFR pathways lead to epithelial neoplasm including those induced by two-stage carcinogenesis in mouse skin where activation of Ha-Ras is a critical event in papilloma formation (Frame et al., 1998). Topical application of diverse tumor promoters on mouse skin leads to elevated levels of EGFR and its ligands TGF-α, HB-EGF, and AR in developing primary papillomas and SCC (Kiguchi et al., 1998). Interestingly, overexpression of TGF-α in basal or suprabasal keratinocytes leads to thickening of the epidermis and papilloma development preferentially at sites exposed to mechanical irritation, and TGF-α expression can bypass the need for chemically induced Ha-Ras mutations (Vassar et al., 1992; Dominey et al., 1993; Wang et al., 1994). Furthermore, overexpression of ErbB2 in basal cells of the epidermis results in the formation of spontaneous papillomas capable of converting to SCC within the first 6 weeks of age (Kiguchi et al., 2000). Constitutive expression of an activated form of Ha-Ras in the suprabasal layers of the epidermis induces the formation of benign papillomas in transgenic mice at sites of promotional stimuli (Bailleul et al., 1990). However, transgenic mice expressing an activated Ras in the outer root sheaths of hair follicles develop spontaneous papilloma-like structures, which frequently undergo conversions to SCC (Brown et al., 1998). In contrast, tamoxifen-inducible activation of a K14-Ras transgene in mice results in massive cutaneous hyperplasia and suppressed differentiation that is reversible upon cessation of tamoxifen treatment (Tarutani et al., 2003).

An elegant *in vivo* demonstration on the role of EGFR in the early steps of skin tumor development was provided by the analysis of transgenic mice expressing a constitutively active form of the Ras activator Son of Sevenless (SOS) in the basal cells of the epidermis (K5-SOS-F mice, Table 2). These mice develop skin papillomas at 100% penetrance in a wt EGFR background (Fig. 3F). However, tumor formation is severely impaired when these mice are bred into an EGFR mutant background (Table 2). K5-SOS-F transgenic papillomas and keratinocytes from wa2 mice are more differentiated and display increased apoptosis as well as

reduced Akt phosphorylation, suggesting that the EGFR functions as a survival factor for oncogenic transformation by components of the Ras signaling pathway (Sibilia et al., 2000). In mice lacking c-Jun in the epidermis, K5-SOS-F-dependent skin tumorigenesis is strongly inhibited and accompanied by reduced expression of EGFR in basal keratinocytes (Table 2). In this study, it could be shown that Jun regulates EGFR expression at the transcriptional level (Zenz et al., 2003). Recently, it was shown that patients with dominant Hereditary Gingival Fibromatosis type 1 carry a frameshift mutation in the SOS1 gene, leading to a truncated SOS protein similar to the one expressed in K5-SOS-F mice (Hart et al., 2002), highlighting the relevance of K5-SOS-F transgenic mice as a model for human cancer and for testing anti-tumor therapies.

Interestingly, EGFR-deficient fibroblasts are also resistant to transformation by SOS-F and RasV12 (Sibilia et al., 2000). An independent study demonstrates that EGFR-deficient keratinocytes expressing v-ras^{Ha} develop smaller papillomas when grafted into immunodeficient mice (Dlugosz et al., 1997). Similar results were obtained with Ha-Ras transgenic animals expressing dominant-negative EGFR (K5-dnEGFR) in basal keratinocytes. Whereas tumor onset is similar to controls in mice expressing dnEGFR, tumors appear much paler and their size is reduced (Casanova et al., 2002). Interestingly, these papillomas show increased numbers of apoptotic cells and reduced Akt activity. Furthermore, abrogation of EGFR function results in a dramatic decrease in vascular endothelial growth factor (VEGF) expression and impaired angiogenesis. As a consequence, tumors cannot be properly nourished and oxygenated, which may explain the poor cell survival. These findings provide compelling functional evidence that, in addition to the Ras/MAPK pathway, an EGFR-dependent pathway acting via Akt is essential for the transformation of mouse epidermal cells (Segrelles et al., 2002). Whether a similar mechanism is acting in human SCC needs to be investigated.

Recent studies show that mitogen-inducible gene 6 (Mig6, also known as RALT) is a specific negative regulator of EGFR signaling in skin morphogenesis. Mice deficient for Mig6 display hyperactivation of endogenous EGFR, resulting in overproliferation and impaired differentiation of epidermal keratinocytes (Table 2). Furthermore, Mig6$^{-/-}$ mice spontaneously develop tumors in various organs and are highly susceptible to chemically induced skin tumor formation. Interestingly, breeding of Mig6 knock-out mice into an EGFR hypomorphic background or treatment with Gefitinib completely rescues the skin defects and tumor development is prevented. Therefore, Mig6 acts as a tumor suppressor in EGFR-dependent carcinogenesis and its expression is downregulated in various human cancers (Ballaro et al., 2005; Ferby et al., 2006). The transcription factor AP-2α is also often reduced in tumor cells and was recently shown to act as a tumor suppressor (Friedrichs et al., 2005). Deletion of AP-2α in mice results in a similar phenotype as Mig6$^{-/-}$ mice. In the absence of AP-2α, EGFR expression is up-regulated and Akt activation is increased in the epidermis, resulting in hyperproliferation and the formation of papilloma-like invaginations. These data indicate that AP-2α controls epidermal cell proliferation and differentiation, and functions by repressing EGFR expression at the stage when keratinocytes exit the basal layer and become committed to terminal differentiation (Wang et al., 2006).

Altogether, these findings underscore the importance of EGFR as a target for therapeutic intervention in epithelial tumors. Because it has been shown that EGFR signaling pathways are frequently deregulated in human skin cancers by mutations of tumor suppressors such as Mig6 and AP-2α, in the future it would be interesting to consider these negative regulators as new targets for therapeutic interventions.

EGFR in lung development and cancer

At birth, EGFR mutant mice have immature lungs resembling a disease known as neonatal respiratory distress syndrome. This defect is most likely responsible for the inability of the majority of mutants to initiate or sustain respiration. Mutant mice display lung dysplasia characterized by lung hypercellularity and thickened alveolar septae, leading to reduced airspace in the lungs (Miettinen et al., 1995, 1997; Sibilia and Wagner, 1995; Sibilia et al., 1998). Lung branching morphogenesis is defective in EGFR mutants, leading to deficient alveolization and septation already during embryogenesis (Miettinen et al., 1997). EGFR is expressed on type II pneumocytes and seems to regulate their maturation by inducing the expression of surfactant protein C and thyroid transcription factor-1 (Sibilia and Wagner, 1995; Miettinen et al., 1997). The lung defects can be ameliorated by inducing lung maturation via dexamethasone administration, which also improves the early postnatal survival of EGFR mutant mice. However, the maximal lifespan of mutant mice is not increased and there is also no amelioration of the other phenotypes (Sibilia et al., 1998).

Lung tumors account for 1/3 of cancer deaths worldwide and can be divided into two main groups: (1) small-cell-lung cancer (SCLC), which originates from neural crest cells and comprises about 20% of lung tumors, and (2) non-small-cell lung cancer (NSCLC) making up 80% of all lung cancers and deriving from epithelial cells. Among the many different types of NSCLC, one can distinguish adenocarcinomas originating most likely from pneumocytes type II and bronchioloalveolar carcinomas, a subtype of adenocarcinoma with

better prognosis derived either from bronchio-alveolar stem cells or representing a more benign stage of invasive adenocarcinomas (Sharma et al., 2007). The most frequent mutations found in NSCLC affect K-Ras or EGFR, both occurring in a mutually exclusive manner, with EGFR mutations arising prevalently in non-smokers (Pao et al., 2005b). EGFR ligands like TGF-α and EGF are frequently expressed in NSCLC and EGFR overexpression has been reported in over 60% of NSCLC, correlating with a poor prognosis. The median survival time of patients is around 4–5 months and is only modestly extended by standard combination chemotherapy (Sharma et al., 2007).

A few years ago, the reversible EGFR inhibitors Gefitinib (Iressa) and Erlotinib started to be used for the treatment of NSCLC that had failed to respond to conventional chemotherapy. It was found that a subpopulation of 10%–20% of patients responded very well to the therapy with significant regression of the tumors. Interestingly, 80% of these patients are carrying somatic mutations within the EGFR kinase domain in tumor cells, the most frequent being either deletion of the conserved LREA motif within Exon 19 or a point mutation in Exon 21 (L858R). Both these mutations are gain-of-function mutations and occur more frequently in females, Asians, and in non-smokers (Lynch et al., 2004; Paez et al., 2004; Pao et al., 2004). It is intriguing that these EGFR mutations are prevalently restricted to NSCLC as they seem to occur only at a very low frequency in other tumors such as head and neck, ovarian, pancreatic, and colorectal cancers (Sharma et al., 2007). *In vitro* studies with NSCLC cell lines carrying EGFR kinase mutants reveal that the Akt, ERK, STAT 3, and STAT 5 pathways are hyperactivated, resulting in increased survival and reduced apoptosis of tumor cells. These effects can be reversed by treatment with Gefitinib or Erlotinib (Sordella et al., 2004; Sharma et al., 2007).

The oncogenicity of these Gefitinib-sensitive EGFR mutations was recently demonstrated in transgenic mouse models. Mice expressing either form of the mutated EGFR in a doxycycline-inducible manner in type II pneumocytes develop invasive lung adenocarcinomas, recapitulating many common features to human tumors (Ji et al., 2006; Politi et al., 2006). If mice are withdrawn from doxycycline, EGFR levels rapidly decline and established tumors disappear within 3 weeks, showing a strong decrease in proliferation and an increase in apoptosis. A significantly reduced tumor burden was also observed after treatment with Erlotinib and to a lesser extent with the EGFR-specific antibody Cetuximab (Erbitux) (Ji et al., 2006; Politi et al., 2006).

These promising results obtained in mice do not completely hold true when treating NSCLC patients. Patients harboring TKI-sensitive EGFR mutations initially respond to Gefitinib and Erlotinib with stabilization rather than shrinkage of the tumors. However, resistance to TKI treatment usually occurs within 6–12 months therefore limiting the ability of these drugs to improve the patient's survival significantly. Tumors acquire secondary resistance to both Gefitinib and Erlotinib by additional point mutations in the EGFR at position 790 within the kinase domain (Kobayashi et al., 2005; Pao et al., 2005a). Interestingly, this T790M mutation weakens the interaction of the inhibitor with the kinase and is analogous to other drug-resistance-conferring mutations found in BCR-Abl, PDGFRα, and c-Kit, suggesting that cancer cells depend on a similar cellular pathway for survival or proliferation (Sharma et al., 2006, 2007).

The overall survival rate of NSCLC patients is slightly improved only under Erlotinib treatment, explaining why Gefitinib has meanwhile been removed from the market. New irreversible ErbB inhibitors such as HKI 272 and HKI 357 seem to be able to overcome the Gefinitib resistance conferred by the T790M mutation. These specific inhibitors of EGFR and ErbB2 act by covalently binding cysteine residues and can also be more effective in some TKI-insensitive NSCLC. Interestingly, this new class of inhibitors is also active against the small fraction of NSCLC expressing the EGFRvIII mutant, which is insensitive to Gefitinib and Erlotinib. Moreover, the capacity of some of these inhibitors to also target ErbB2 might additionally contribute to overcome resistance to EGFR inhibitors and improve the therapeutic outcome (Kwak et al., 2005; Sharma et al., 2007).

EGFR in liver development and hepatocellular carcinoma formation

Hepatocytes of the mature liver express high amounts of EGFR when compared with other adult cells and tissues, implying that EGFR plays an important role in liver function. Moreover, EGFR ligands like EGF, TGF-α, AR, and HB-EGF are potent mitogens for cultured hepatocytes (Michalopoulos and DeFrances, 1997; Fausto, 2000) and significantly contribute to liver regeneration after partial hepatectomy as demonstrated in the corresponding knock-out mice (Russell et al., 1996; Berasain et al., 2005; Mitchell et al., 2005). Also, during acute and chronic liver damage like CCl_4 intoxication and Fas-mediated liver injury, EGFR ligands seem to have a hepatoprotective and regenerative potential (Berasain et al., 2007). Studies from our laboratory using conditional knock-out mice show that inducible deletion of the EGFR in all liver cell types of adult mice does not lead to overt abnormalities. In contrast, embryonic deletion of EGFR in hepatocytes alone results in reduced body size and weight, which is apparent from the third postnatal week. Moreover, mice lacking EGFR in liver show impaired liver regen-

eration after partial hepatectomy with reduced cyclin D1 expression. These results demonstrate that EGFR is a critical regulator of hepatocyte proliferation in the initial phases of liver regeneration (Natarajan et al., 2007).

Many growth factors and their receptors are deregulated in human hepatocellular carcinoma (HCC). HCCs account for 83% of cases of liver cancer, which include cholangiocarcinoma, hepatoblastoma, bile duct cystadenocarcinoma, hemangiosarcoma, and epitheliod hemangioendothelioma (Farazi and DePinho, 2006). HCCs are complex heterogeneous neoplasms, with an 8.9% 5-year survival rate, therefore recording this malignancy as the second most lethal cancer following pancreatic ductal adenocarcinoma (Breuhahn et al., 2006; Farazi and DePinho, 2006). Various etiological factors associated with the development of HCC include chronic hepatitis B and C viral infection, chronic alcohol consumption, aflatoxin B1 in food, and virtually any other condition that leads to cirrhosis (Badvie, 2000). Among the key signaling systems believed to play a prominent role in the development of HCCs are TGF-α /EGFR, IGF/IGF-1R, and HGF/Met (Farazi and DePinho, 2006). Most of these growth factors, receptors, and signaling cascades are also involved in regenerative and protective natural responses of the liver to acute tissue injury. However, when these growth factors are deregulated through a series of molecular events, they can contribute to neoplastic transformation (Thorgeirsson and Grisham, 2002; Berasain et al., 2007). Other genes that have been reported to be mutated and aberrantly expressed during HCC are p53, p73, Rb, mdm2, APC, p16, c-myc, cyclin D1, E-cadherin, and gankyrin (Thorgeirsson and Grisham, 2002; Farazi and DePinho, 2006; Laurent-Puig and Zucman-Rossi, 2006).

Examination of the ErbB receptors in human HCCs indicated that overexpression of EGFR occurs in 68%, ErbB2 in 21%, ErbB3 in 84%, and ErbB4 in 61% of HCCs (Breuhahn et al., 2006). Increased levels of ErbB1 and ErbB3 expression have been associated with aggressive tumors and poorly differentiated HCCs. Metastasis and poor patient survival is correlated to ErbB1 overexpression (Breuhahn et al., 2006). In addition, overexpression of EGFR ligands such as TGF-α, BTC, HB-EGF, and AR is observed in human liver tumor tissues (Breuhahn et al., 2006; Farazi and DePinho, 2006).

Studies from transgenic mice provide strong evidence for an involvement of the ErbB family of receptors and its ligands in HCCs. The EGFR is highly expressed in chemically induced HCCs in mice (Fig. 3G). Transgenic mice expressing TGF-α from the mouse metallothionein-1 (MT) promoter develop multifocal, well-differentiated HCCs (Table 2) (Jhappan et al., 1990; Sandgren et al., 1990). Treatment of these mice with hepatocarcinogens accelerates the development of HCC, whereas the same treatment only results in small pre-neoplastic foci in TGF-α-deficient mice, highlighting the importance of TGF-α in tumor progression (Webber et al., 1994; Russell et al., 1996). Further evidence that EGFR signaling is vital in HCC comes from pharmacological studies. Inhibition of EGFR by Gefitinib results in growth inhibition, cell cycle arrest, and apoptosis of HCC cell lines and also shows activity in a chemically induced model of HCC in rats (Schiffer et al., 2005; Breuhahn et al., 2006). The anti-EGFR antibody Cetuximab (Erbitux) is also effective in inhibiting cell cycle progression and inducing apoptosis in HCC cells (Breuhahn et al., 2006).

Ectopic expression of the proto-oncogene c-Myc in murine hepatocytes promotes liver tumor development and targeted inactivation of c-Myc in mice results in tumor regression (Shachaf et al., 2004). Co-expression of TGF-α and c-Myc in murine liver accelerates tumor development when compared with the respective single transgenic mice (Table 2) (Jhappan et al., 1990; Murakami et al., 1993). This synergistic effect on HCC in double transgenic mice is due to the disruption of the E2F/pRB pathway and reduced apoptosis (Santoni-Rugiu et al., 1998). Moreover, constitutive nuclear translocation of NF-κB with aberrant activation of Akt and IKK complex is observed in HCCs of TGF-α/c-Myc double transgenic mice, but not of c-Myc single transgenic mice (Arsura and Cavin, 2005). This study suggests that an anti-apoptotic mechanism mediated via the TGF-α-Akt-IKK pathway contributes to survival and proliferation, thereby disabling the apoptotic pathways induced by transforming oncogenes such as Myc (Arsura and Cavin, 2005). Inactivation of the AP1 transcription factor c-jun in the early stages of liver tumor development results in reduced tumorigenesis, accompanied by increased levels of p53, which leads to increased tumor cell apoptosis (Eferl et al., 2003). Because c-Jun is known to regulate skin tumor development by regulating EGFR expression (Zenz et al., 2003), it would be interesting to investigate whether c-Jun controls liver tumor development in a similar manner.

Overexpression of the Met receptor and its ligand HGF has been reported in advanced human HCC (Thorgeirsson and Grisham, 2002; Breuhahn et al., 2006). The role of Met signaling in HCC is further confirmed by transgenic mice expressing HGF that develop HCC (Sakata et al., 1996). Inducible Met transgene expression in livers results in HCCs, and inactivation of the transgene in advanced tumors leads to tumor regression (Wang et al., 2001). Furthermore, the association of c-Met with EGFR in HCC cells facilitates the activation of c-Met in the absence of HGF. Neutralizing antibodies against TGF-α or EGFR abrogate c-Met phosphorylation, suggesting that there is crosstalk between EGFR and c-Met in transformed cells (Jo et al., 2000). Thus, anticancer therapies

targeting EGFR components might be even more efficacious in HCC treatment as they might also inhibit the c-Met signaling pathway.

EGFR in bone development and bone cancer

Understanding the role of the EGFR in skeletal development and bone cell differentiation is a pre-requisite for understanding the influence of the EGFR in bone cancer. Compelling evidence for the involvement of EGFR in bone development is provided by the analysis of mice lacking the EGFR and mice humanized for the EGFR (Sibilia et al., 2003; Wang et al., 2004). $hEGFR^{KI/KI}$ mice are significantly smaller than their control littermates and the zone of hypertrophic chondrocytes is increased within the growth plate of their long bones, suggesting that EGFR signaling negatively regulates the maturation of hypertrophic chondrocytes (Sibilia et al., 2003). This hypothesis is supported by *in vitro* findings from cultures of mesenchymal cells derived from chicken limb buds where EGF inhibits chondrogenesis (Yoon et al., 2000). In another study using EGFR-deficient mice, it was demonstrated that insufficient vascularization and delayed osteoclast recruitment into $EGFR^{-/-}$ cartilage during embryonic development may be responsible for impaired endochondral bone formation (Wang et al., 2004). EGFR expression on osteoclasts is controversial and further studies are needed to determine whether osteoclast formation depends directly on EGFR signaling (Tanaka et al., 1998; Wang et al., 2004; Normanno et al., 2005). In contrast to osteoclasts, there is general agreement that the EGFR is expressed on mesenchymal cells including osteoblasts and chondrocytes (Chien et al., 2000; Sibilia et al., 2003; Wang et al., 2004). EGFR-deficient osteoblasts have been shown to differentiate much faster, thereby losing their ability to proliferate (Sibilia et al., 2003). Although the molecular mechanisms underlying this defect have not yet been determined, the defects of $EGFR^{-/-}$ osteoblasts observed *in vitro* may also account for impaired bone formation *in vivo* in mice. Most importantly, EGFR signaling in human osteoblasts may play a central role in bone cancer and bone metastasis.

In human osteosarcomas, an increase in ErbB2 expression seems to correlate with a poor clinical outcome. However, another report shows an association of increased ErbB2 expression with improved patient survival (Wen et al., 2007). In contrast to ErbB2, only limited data on EGFR expression in human osteosarcomas are available. These studies show EGFR expression in 57%–81% of the analyzed tumors. (Oda et al., 1995; Wen et al., 2007). It has long been known that mice overexpressing c-fos develop osteosarcomas (Ruther et al., 1989). A recent study shows that the growth factor-regulated S6 kinase Rsk2 plays a central role in Fos-dependent osteosarcomas, because mice overexpressing c-Fos, but lacking Rsk2 have significantly impaired tumor development (David et al., 2005). Interestingly, EGFR signaling can induce the transcription of the c-fos gene via Rsk2 (De Cesare et al., 1998). Considering that osteosarcomas are tumors affecting the osteoblastic lineage and knowing that the EGFR controls osteoblast function, it seems reasonable to speculate that there might be a direct relationship between EGFR signaling and c-Fos-mediated osteosarcoma development. Consequently, it is important to determine whether EGFR and c-Fos indeed act in concert in inducing and/or promoting osteosarcoma, and whether this interaction may also occur in human osteosarcomas or in other types of tumors. For example, besides human osteosarcoma and osteosarcoma cell lines, EGFR upregulation is observed in bone- and soft-tissue tumors (Dobashi et al., 2007) and also in ameloblastoma, the most common epithelial odontogenic tumor of the jawbones. In these tumors, which originate from EGFR-expressing odontogenic epithelium, EGFR protein expression is detected in all specimens analyzed (Vered et al., 2003).

The role of EGFR in bone metastasis is worth investigating as EGFR expression has been reported in a variety of tumors metastasizing to the bone and, among them, prostate cancer is the best-studied example (Choueiri et al., 2006). Forty percent to 80% of prostate cancers express the EGFR correlating with decreased disease-free survival (Di Lorenzo et al., 2002; Herbst and Langer, 2002). Prostate carcinoma cells preferentially metastasize to bone where they typically cause early osteoblastic and late osteolytic lesions (Choueiri et al., 2006). The central role of osteoblasts in the metastasis of prostate cancer may be linked to EGFR expression, not only because prostate cancer cells secrete EGF but also because osteoblast expression of bone matrix proteins such as osteopontin, which stimulate prostate cancer cells, seems to be EGFR dependent (Soulitzis et al., 2006). Moreover, Gefitinib treatment reduces the incidence of prostate cancer metastases of highly metastatic prostate cancer cell lines in nude mice by 81% (Angelucci et al., 2006). In addition, EGFR inhibition with PKI166, a novel EGFR TKI, also significantly reduces the incidence of bone metastasis in nude mice (Kim et al., 2004). Other than prostate cancer metastases, human renal cell carcinoma bone metastases are also significantly reduced in nude mice treated with PKI166 (Weber et al., 2003). Interestingly, in a phase II trial of breast cancer patients with bone metastases, administration of Gefitinib leads to significant relief of bone pain (Normanno et al., 2005). Because the main mechanism in bone destruction by breast cancer cells is tumor-mediated stimulation of osteoclastic bone resorption (Roodman, 2002), the above mentioned controversy about EGFR expression

on osteoclasts remains an issue important to be addressed.

Conclusions and perspectives

From the analysis of mice genetically modified for EGFR expression, it seems that the EGFR controls different processes in different tissues and cells. In cortical astrocytes and epidermal tumors, the EGFR seems to act as a survival signal via inducing the anti-apoptotic Akt pathway. In the epidermis, EGFR signaling probably also prevents premature differentiation of keratinocytes as K5-SOS-F-dependent skin tumors are more differentiated in the absence of EGFR. Similarly, the EGFR also seems to negatively affect osteoblast and chondrocyte differentiation, which inversely correlates with the proliferation capacity of these cells. In contrast, the EGFR is required for efficient differentiation of mesenchymal cells in the semilunar valves of the heart and of type II pneumocytes in the lung whereas in hepatocytes the EGFR is required for cell proliferation and cell-cycle entry following tissue injury. Furthermore, the EGFR controls both proliferation and survival of tumor cells in lung adenocarcinomas induced by transgenic expression of TKI-sensitive EGFR mutants.

From these results, it is evident that the cellular processes controlled by the EGFR are very complex and that we are just starting to understand them. There are many unanswered questions and the intricate EGFR-dependent signaling network needs to be elucidated further in every cell and organ to understand the physiological function and pathways controlled by the EGFR. This knowledge will be instrumental to gain insights into the role of EGFR during tumor initiation, progression, and metastasis. For example, it is still unclear whether EGFR overexpression or mutations play any role in the early phases of malignant transformation or whether EGFR alterations are rather secondary consequences of a selection process leading to the growth advantage of already established tumors. One additional issue to clarify is whether EGFR mutations affect the various stages of tumor development in a different way than for example amplification or overexpression. This knowledge will be essential to develop rational and more effective therapies for the treatment of human cancer.

We also need better mouse models where we can test these concepts and perform relevant *in vivo* biological and preclinical studies. Except for lung adenocarcinomas, there are not many good mouse models recapitulating the hallmarks of the equivalent human cancer. Although our K5-SOS-F transgenic mouse model does not fully resemble the human disease, it allows investigating the early events leading to epithelial cell transformation. In this model, efficient cellular transformation and tumor formation by components of the Ras signaling pathway requires an intact EGFR signaling pathway. These findings establish an important role for EGFR in cell survival in the early phases of oncogenic transformation and indicate that EGFR inhibitors can not only be applied for the therapy of tumors with EGFR alterations but also against tumors carrying activating mutations of the Ras oncogene. K5-SOS-F transgenic mice are extremely useful for preclinical validations, because tumor incidence is 100% and occurs within the first 2 months after birth in an immunocompetent environment. In addition, tumor growth is fast and can be monitored very easily without sacrificing the animals and without the need for sophisticated imaging devices. For these reasons, K5-SOS-F mice can be instrumental in identifying additional genetic alterations that are responsible for tumor formation and that represent specific targets for therapeutic intervention.

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6 MATERIALS AND METHODS

The materials and methods listed below belong to the results described in 5.1. More information can be found in the "Materials and methods" sections of the manuscripts "Autocrine VEGF signaling synergizes with EGFR in tumor cells to promote epithelial cancer development" and "Conditional deletion of β1 integrin impairs SOS-dependent skin tumor development in transgenic mice", respectively.

6.1 Mouse strains

Conditional EGFR mice ($EGFR^{f/f}$) (Natarajan et al., 2007) were crossed to K5-Cre (Tarutani et al., 1997) and K5-$CreER^{T}$ (Indra et al., 1999) transgenic mice to generate mice in which EGFR is constitutively deleted in the basal layers of the epidermis starting from embryonic day 14.5 ($EGFR^{\Delta ep}$), or mice in which EGFR deletion could be induced by administration of tamoxifen ($EGFR^{\Delta epER}$), respectively.

Hairless (hr/hr) mice were purchased from Harlan. $EGFR^{-/-}$ (Sibilia and Wagner, 1995) and $hEGFR^{KI/KI}$ mice (Sibilia et al., 2003) were available in the laboratory. Mice were kept in the animal facility of the Medical University of Vienna in accordance with institutional policies and federal guidelines.

6.2 Tamoxifen treatment

To induce EGFR deletion K5-$CreER^{T}$ transgenic $EGFR^{f/f}$ mice were injected intraperitoneally with 1mg of tamoxifen per 25g body weight (Sigma; sunflower seed oil/ethanol mixture (10:1) at 10mg/ml) per day on 5 consecutive days and then twice a week for maintenance.

For the wound healing assay, in addition to the i.p. injection of tamoxifen, 4 mg of 4-hydroxy-tamoxifen (Sigma) dissolved in 0.2 ml Acetone was applied to the backskin of $EGFR^{\Delta epER}$ and control mice every other day for a total of 2 weeks before full thickness punch wounds were made. Deletion efficiencies were analyzed by Southern and Western blot analysis.

6.3 Isolation of genomic DNA

3-5 mm of mouse tail, small pieces of tissue or cell pellets were incubated overnight in 0.5 ml of lysis buffer (50mM Tris pH 7.4, 100mM EDTA, 100mM NaCl, 1% SDS and 0.5 mg/mL Proteinase K) at 55°C. 250µl of 6M NaCl were added, the suspension mixed and centrifuged for 5 minutes at full speed (13500 rpm). The supernatant was transferred to a fresh tube and the DNA was precipitated with 0.5mL isopropanol and pelleted by centrifugation. After washing with 70% ethanol the DNA was resuspended in 0.4mL TE (10mM Tris pH 7.6, 1mMEDTA). 1µl and 49µl of this DNA solution were used for PCR and Southern blot analysis, respectively.

6.4 Genotyping of transgenic mice by PCR

1µl of genomic DNA solution was used to analyse the genotype of transgenic mice. The following primers were used: to distinguish wild-type, floxed and flirt EGFR alleles: FLIRT 2 5'-ATCAGCAGCCTCTGTTCCACATACAC-3', FRT4 5'-CTATGCCTAAGAGGCGGAATA-3', and FLIRT5 5'-GACCATAGGAGGAACTGGACG; and to detect the K5-Cre or K5-CreER^{T} transgenes: K5Cre1 5'-CATACCTGGAAAATGCTTCTGTCC-3' and K5Cre2 5'-CATCGCTCGACCAGTTTAGTTACC-3'. The EGFR flirt allele is a floxed allele containing a Neo-cassette. Since it behaves exactly like the EGFR floxed allele, both alleles were indicated as EGFR$^{f/f}$ for simplicity.

6.5 Genotyping of transgenic mice by Southern blot analysis

5 to 10 µg of genomic DNA were digested with 20U of Hind III restriction enzyme and separated by electrophoresis through a 0.8% agarose gel. The DNA was depurinated in 0.25M HCl, denatured in 0.5M NaOH/1.5M NaCl and transferred to a nylon membrane (Gene Screen, Du Pont). After the transfer, the membrane was washed in 50 mM Na-phosphate and UV crosslinked. Hybridizations with radioactive probe were performed at 65°C in Church buffer (0.5M Na-phosphate pH 7.2, 7% SDS) overnight. Thereafter, membranes were washed at 65°C twice for 30 minutes in 40mM Na-phosphate pH 7.2, 1% SDS and exposed to Kodak XAR films at -80°C using intensifying screens.

6.6 Western blot analysis

Protein lysates were prepared as previously described (Sibilia et al., 2000) and proteins were separated by SDS-PAGE and transferred to PVDF membranes (Millipore). Western blot analysis was performed as previously described (Sibilia et al., 2000) with antibodies detecting EGFR (Upstate Biotechnology) and actin (Sigma).

6.7 Isolation & culture of mouse keratinocytes

Shaved mice were skinned and the subcutaneous tissue was scraped off. Small pieces of the skin were placed hairy side up onto 0.8% Trypsin/PBS (Invitrogen) and incubated 45 min at 37°C. Epidermis was separated from dermis and placed into low calcium medium (Sigma) containing 8% chelated FCS and 250µg/mL DNAse (Sigma). After 20 min shaking in a 37°C water bath, the suspension was filtered through a 70µm cell strainer (Becton Dickinson). Keratinocytes were seeded at a density of $6x10^6$ cells/100mm dish onto vitrogen-fibronectin coated dishes in low calcium medium (Sigma) containing 8% chelated FCS and several other factors (Carroll et al., 1995) and incubated at 32°C, 5% CO_2.

6.8 Histological analysis

Skin biopsies were fixed in 4% paraformaldehyde, embedded in paraffin and cut into 5µm sections. Prior to staining, sections were dewaxed in xylene and re-hydrated through a series of ethanol dilution (100%, 95%, 90%, 80%, 70% and 30%) and incubated in water for 10 minutes. Rehydrated sections were stained with Harris haematoxylin and eosin (H&E, Sigma) according to standard procedures. Giemsa and Fontana Masson stainings were also performed according to standard procedures. Images were obtained with a Nikon eclipse 80i microscope; histomorphometric analysis was perfomed with Lucia software. Staging of hair follicles was performed according to the guide for the recognition and classification of distinct stages of hair follicle morphogenesis (Paus et al., 1999).

6.9 Dye exclusion assay

Embryos were incubated for 1 min in 25, 50, and 75% methanol in PBS, followed by a 1 min incubation in 100% methanol, and a descending series of incubations in 75, 50, and 25% methanol in PBS for 1 min. Embryos were then washed in PBS for 1 min and stained with 0.1% toluidine blue O (Sigma) for 10 min.

6.10 Wound healing assay

To address the function of EGFR in wound healing, 5mm full thickness punch wounds were placed with sterile punching devices at the back of adult tamoxifen-treated $EGFR^{\Delta epER}$ mice and their littermate controls, and of $hEGFR^{KI/KI}$ and their wild-type littermates, as well as of 3.5-week old $EGFR^{\Delta ep}$ mice and their controls. Skin biopsies were isolated at different time points after injury and processed for histological analysis.

6.11 Flow cytometric analysis

Cell suspensions from epidermis and dermis were isolated as described above and filtered through a 70µm nylon mesh, counted and stained with mAbs for 30 min in PBS + 5% FCS at 4°C after blocking with Fc-block (BD Pharmingen). The following mAbs were used: anti-CD3ε-PE (clone145-2C11), anti-CD11c-FITC (clone HL3), anti-CD45-APC (clone 30F11), anti-MHCII-FITC (clone 2G9), all from BD Pharmingen; and anti-CD4-TC (clone RM4-5), anti-CD8α-Alexa 647 (clone 5H10), anti-CD11b-FITC (clone M1/70.15) and anti-Ly6C/G (clone RB6-8C5), all from CALTAG laboratories. Dead cells were excluded by adding 7AAD (Sigma) at a final concentration of 1µg/mL after the last washing step.

Popliteal, inguinal, brachial, axillar and retroauricular lymph nodes were pooled, minced and filtered through a 70µm nylon mesh, counted and, after blocking, stained with the following antibodies: Anti-CD4-PE-Cy7 (clone RM4-5), anti-CD25-biotinylated (clone PC61, both BD Biosciences) and anti-CD8α (clone 52-6.7, Biolegend). Cells were washed twice with PBS/FCS and fixed in 2% paraformaldehyde over night. The next day cells were washed and the cell pellet was resuspended in 1x Perm/Wash (BD Biosciences), pelleted and again

resuspended in 500µLof 1x Perm/Wash. After 15 min of permeabilization at room temperature the cell suspension was centrifuged and cells were resuspended in 50µLPerm/Wash. For intracellular staining the following antibiodies were added: anti-FoxP3-A647 (clone150D, Biolegend) or mouse IgG1κ (clone MOPC-21) as an isotype control. Cells were stained 30 minutes on ice and washed once in Perm/Wash and 2x in PBS/FCS before acquisition on the flow cytometer. Data were acquired on a LSR-II flow cytometer (BD Biosciences) and analyzed with CellQuest software (BD Biosciences).

6.12 Statistical methods

All experiments were repeated at least twice and done in triplicates. Data were evaluated using a Student's two-tailed t test. $p<0.05$ was taken to be statistically significant. In Figures 9F and 11F data were analyzed by a Log-rank (Mantel-Cox) test.

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8 CURRICULUM VITAE

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PUBLICATIONS

The epidermal growth factor receptor: from development to tumorigenesis. Sibilia M., Kroismayr R., Lichtenberger B.M., Natarajan A., Hecking M., Holcmann M.; *Differentiation. 2007 Nov;75(9):770-87.*

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