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„The Role of Epidermal Growth Factor Receptor in Inflammation and Tumorgenesis“

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Abstract

The proper expression of the epidermal growth factor receptor (EGFR) on the cell surface as well as its appropriate signal transduction in different organs and cell types is determining prenatal and postnatal development. The EGFR signaling network is essential for skin development and its appendages, as well as tumor formation and progression, respectively. EGFR is one of the key receptors in keratinocytes controlling proliferation, differentiation, migration and most notably inflammatory responses, cellular processes which play an essential role in tumor formation and progression.

Due to the fact that EGFR-null mice die early after birth or during embryogenesis the investigation of EGFR's function in skin inflammation and carcinogenesis has been difficult. However, this issue was avoided when $EGFR^{fl/fl}$ mice are crossed to K5-Cre and K5-$CreER^{T}$ (Indra et al., 1999)(Indra et al., 1999)transgenic lines to generate mice, where the receptor is already deleted in the skin during embryogenesis ($EGFR^{\Delta ep}$), and mice, where EGFR deletion can be induced postnatally ($EGFR^{\Delta epER}$).

Epidermis-specific deletion of EGFR during embryogenesis results in both a delay in hair follicle cycle as well as an increased skin inflammation. I could demonstrate that depending on the different stages of HF cycle macrophages, myeloid granulocytes, monocytes and dendritic cells are significantly increased compared to litter mate controls. However, I could not determine a significant difference in the number of Langerhans cells and T-cells in the dermis and epidermis lacking EGFR. These findings demonstrate once more, that the expression of EGFR by keratinocytes is essential for inflammatory processes and loss of function causes chronic skin inflammation. The immune system, which acts primarily as a defense against injury and infection, may lead to tumor formation if it is beyond control. The EGFR, participating in the skin immune response, is often overexpressed in human carcinomas and glioblastomas. Previous studies confirmed mesenchymal-epithelial alterations and increased inflammation by abrogation of EGFR function in the skin. To address the mechanisms by which the loss of EGFR signaling pathway leads to increased inflammation and further on to tumor formation, TPA was applied. I revealed that EGFR deficiency reduces TPA-dependent hyperplasia as well as epidermal proliferation and apoptosis. Surprisingly, TPA-dependent ERK1/2

activation seems to be EGFR dependent, whereas NFκB and p38 protein activation seemed to be EGFR independent. Moreover, EGFR deficiency increases TPA-dependent attraction of monocytes, early hematopoietic dendritic progenitor cells, T-cells and dendritic cells. However, I could show that the basic inflammation found in EGFR mutants is not sufficient to induce and/or promote papillomas. Interestingly, in contrast to what was published before, EGFR mutant mice develop tumors earlier than wt controls when treated according to the 2-stage skin carcinogenesis model. Papillomas from $EGFR^{\Delta epER}$ mice display growth advantage towards those evolving in $EGFR^{fl/fl}$ mice in the early stages of tumor formation but they stop growing and enter tumor stasis at later stages. Furthermore, the composition of immune cell infiltrate of mutant mice in the tumor tissue as well as the surrounding skin is different from those in wild-type.

In conclusion, the role of EGFR in the complex signaling network, which regulates the responses to influence inflammation and tumor development, was demonstrated.

Zusammenfassung

Die korrekte Expression des epidermalen Wachstumsfaktorrezeptors (EGFR) an der Zelloberfläche ebenso wie seine Signaltransduktion in unterschiedlichen Organen und Zelltypen determinieren die pränatale und postnatale Entwicklung. Das Signalnetzwerk des EGFR ist essentiell für die Entwicklung der Haut und ihrer Appendizes sowie für die Tumorentstehung und -progression. Der EGFR ist einer der wichtigsten Rezeptoren der Keratinozyten um die Proliferation, Differenzierung, Migration und vor allem Entzündungsprozesse, die eine essentielle Rolle in der Tumorbildung und -progression spielen, zu kontrollieren.

Die Tatsache, dass EGFR-null Mäuse sehr früh nach der Geburt oder während der Embryogenese sterben, hat die Untersuchungen der Funktion des EGFR in Entzündungen der Haut und in der Karzinogenese erschwert. Dieses Problem wird jedoch umgangen, indem $EGFR^{fl/fl}$ Mäuse mit K5-Cre und K5-$CreER^{T}$ (Indra et al., 1999)(Indra et al., 1999)transgenen Linien gekreuzt werden um Mäuse zu generieren, in denen nur in der Haut der Rezeptor schon während der Embryogenese ($EGFR^{\Delta ep}$) deletiert ist und Mäuse, in denen die EGFR Deletion postnatal induziert werden kann ($EGFR^{\Delta epER}$).

Eine epidermal-spezifische Deletion des EGFR während der Embryogenese verursacht sowohl eine Verzögerung des Haarfollikelzyklus, als auch einen Anstieg an Entzündungsherden der Haut. Ich konnte beweisen, dass abhängig von den unterschiedlichen Phasen des Haarfollikelzyklus Makrophagen, myeloide Granulozyten, Monozyten und dendritische Zellen im Vergleich zu ihren Kontrollen signifikant erhöht sind. Ich konnte jedoch keinen signifikanten Unterschied der Langerhanszellen- und T-Zellenanzahl in der Epidermis und Dermis, denen der EGFR fehlt, feststellen. Diese Entdeckungen bestätigen einmal mehr, dass die Expression des EGFR durch Keratinozyten essentiell für Entzündungsprozesse ist und der Verlust seiner Funktion chronische Entzündungen der Haut verursacht. Das Immunsystem, primär agierend als Schutz vor Beschädigungen und Infektionen, könnte ohne Regulierung zur Tumorbildung führen.

Der EGFR, der an der Immunantwort der Haut beteiligt ist, wird in humanen Karzinomen und Glioblastomen häufig überexprimiert. Vorherige Studien bestätigten mesenchymal-epitheliale Veränderungen und eine verstärkte Entzündung durch die Aufhebung der EGFR Funktion in der Haut. Um die Mechanismen zu beschreiben, in

denen der Verlust des EGFR Signalwegs zur Entzündung und weiters zur Tumorbildung führt, wurde TPA eingesetzt. Ich habe gezeigt, dass das Fehlen des EGFR sowohl die TPA-abhängige Hyperplasie, als auch die epidermale Proliferation und Apoptose reduziert. Widererwarten ist die TPA-mediierte Aktivierung von ERK1/2 EGFR abhängig, wobei die Proteinaktivierung von NFκB und p38 EGFR unabhängig zu sein scheint. Außerdem erhöht das Fehlen des EGFR die TPA-abhängige Anlockung von Monozyten, frühen hämatopoietischen dendritischen Vorläuferzellen, T-Zellen und dendritischen Zellen. Allerdings konnte ich zeigen, dass die basale Entzündung in EGFR Mutanten nicht ausreicht um Papillome zu induzieren und/oder zu fördern. Interessanterweise, im Gegensatz zu früheren Publikationen, entwickeln EGFR mutante Mäuse Tumore früher als ihre Kontrollen, wenn sie nach dem 2-stage skin carcinogenesis Modell behandelt werden. Papillome von $EGFR^{\Delta epER}$ Mäusen zeigen einen Wachstumsvorteil im frühen Stadium der Tumorbildung gegenüber jenen, die sich in $EGFR^{fl/fl}$ Mäusen entwickeln, hören aber in späteren Stadien auf zu wachsen und gehen in eine Tumorstasis über. Zusätzlich ist die Zusammensetzung des Immunzelleninfilrats in mutanten Mäusen sowohl im Tumorgewebe, als auch in der umgebenden Haut verändert verglichen zu der des Wildtyps.

Abschließend lässt sich sagen, dass die Rolle des EGFR in dem komplexen Signalnetzwerk, das die Abläufe der Entzündung und Tumorentstehung beeinflusst, erläutert wurde.

Goal of the study

Mice lacking functional EGFR display defects in the development of the skin and its appendages, and fail to develop a proper hairy coat. Using different transgenic mouse strains, where the EGF (epidermal growth factor) receptor is deleted in the epidermis, I investigated whether EGFR has an impact on skin morphology or its immune cell composition. Furthermore, I addressed whether EGFR signalling is involved in the regulation of hair follicle morphogenesis and the hair cycle as well as the immune cell infiltration in the skin happening in this developmental phase.

Moreover, I aimed elucidating if the skin inflammation induced by the lack of EGFR is sufficient to induce papillomas after initiation with cancerogenic agent. Analysing the apoptosis and proliferation rate, as well as the immune cell infiltration of papillomas and surrounding tissue, I investigated whether EGFR has an impact on tumor morphology and development.

Introduction

1. The skin

1.1 Skin development and appendages

The skin is the interface of the body with the environment. The skin epidermis and its appendages exposed to persistent damage serve as a mechanical, chemical and immunological protective barrier against external influences like bacteria, chemical carcinogens or UV light. To perform these functions a constant process of self-renewal, dependent on stem cells residing in the epidermis, has to be assured. These stem cells have a crucial role in maintaining epidermal homeostasis, hair regeneration and wound healing (Fuchs and Nowak, 2008).

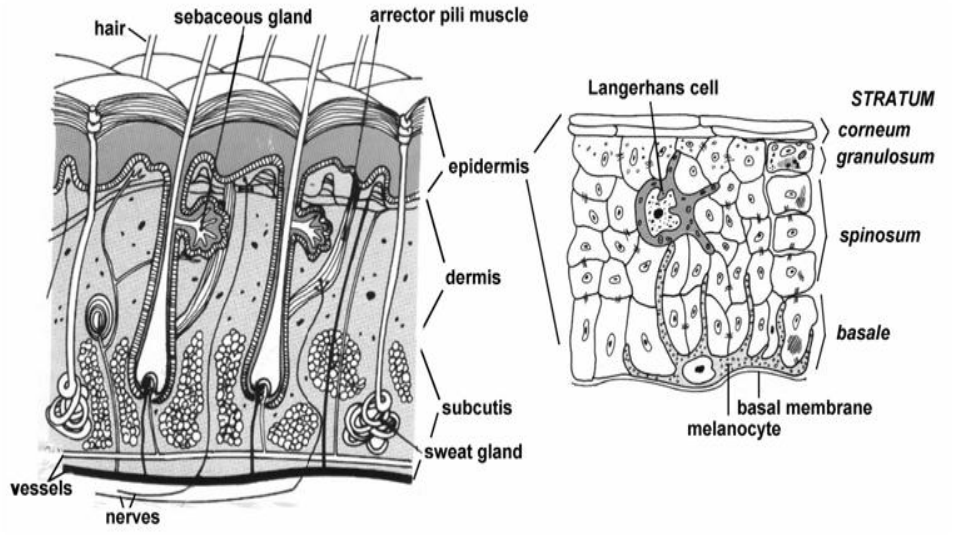


Figure 1 | Skin morphology. Cross-section through mammalian skin.

The majority (95%) of the cells in the single inner (basal) layer of the epidermis, the so called keratinocytes, are fast dividing progeny of stem cells and adhere to an underlying basement membrane (BM). The BM, which separates the epidermis from the underlying dermis, is rich in extracellular matrix (ECM) and growth factors. The keratinocytes in the BM are responsible for the generation of the three nondividing and morphologically and biologically distinct cell layers called spinosum, granulosum and corneum. Undergoing a program of terminal differentiation thereby

moving outwards, the basal epidermal layer immaculately has to balance epidermal proliferation and differentiation to organize the tissue (Fuchs and Nowak, 2008). On the one hand, too little proliferation leads to thinning of the skin and loss of its function, whereas on the other hand too much proliferation may results in psoriasis or cancer. During embryonic development a single cell layer is formed from embryonic day 9.5 (E9.5) to E12.5 and further populated by mesenchymal cells, which transmit signals. These signals orchestrate the stratification of epidermis and the positioning of downgrowths that define the initiation of hair follicle (HF) morphogenesis (Blanpain and Fuchs, 2009). The process of stratification proceeds during E12.5 – E15.5 and is largely completed by E17.5.

In the interfollicular epidermis (IFE) specific stem cells (SCs), differing from those in hair follicles (HFs), are responsible for maintaining normal homeostasis (Ito et al., 2005; Levy et al., 2005). To date, two different models have been described to explain how a single layer of proliferative cells can yield a multi-layered epidermis: First, slow-cycling SCs differentiate in more rapidly proliferating but transiently amplifying (TA) cells that decrease the expression of surface integrins, like β1 and α6 integrin, which furthermore leads to detachment and differentiation. The other model deals with the idea that SCs submit an asymmetric division, where β1 integrin regulation plays an essential role (Clayton et al., 2007; Lechler and Fuchs, 2005). β1 integrin is not known as a specific epidermal SC marker (Ghazizadeh and Taichman, 2001), however, *β1 integrin*-null basal cells fail to maintain proliferative potential *in vivo (Brakebusch and Fassler, 2005)*. During asymmetric division, it is possible that SCs generate a β1-high cell, and a β1-low cell that undergoes terminal differentiation (Rangarajan et al., 2001). Besides keratinocytes, which are the major cell type in the epidermis, various specialized cells including Langerhans cells (LC), melanocytes (MC), Merkel cells (MKC) and lymphocytes are essential for epidermal homeostasis. In vitro studies showed that LCs, which have been distinguished from other antigen-presenting cells by the expression of langerin, are responsible for the uptake and the processing of lipid antigens and microbial fragments representing them to effector t cells (Hunger et al., 2004). Protection against UV radiation induced damage and skin cancer is maintained by MCs by melanin production (Lin and Fisher, 2007). MKCs, located in the basal layer of the epidermis and the epithelial sheath of hair follicle, form synaptic junctions with dermal sensory axons (Kanitakis,

2002). Furthermore, lymphocytes, mainly $CD8^+$ T cells, can be found in the stratum basale and stratum spinosum (Krueger and Stingl, 1989).

In contrast to the epidermis, the dermal architecture and histology is much more complex. The dermis is composed of collagen, elastic tissue and reticular fibres. Moreover, lymphatic and vascular vessels, which play an important role in preserving cell migration, as well as nerves, are located in the dermis. It also contains many specialized cells, such as dermal DCs and plasmacytoid DCs (pDCs), CD4+ T helper 1 (TH1), TH2 and TH17 cells, γδ T cells and natural killer T (NKT) cells. In addition, macrophages, mast cells and fibroblasts are present (Nestle et al., 2009).

Another important function of keratinocytes, besides migration and adhesion, is the transduction of signals from multiple pathways to regulate their proliferation rate. This can be mediated through the ability of intergrins to activate the Src family tyrosine kinases, which are activators of the Ras-mitogen-activated protein kinase (MAPK) signaling cascade (Lorenz et al., 2007; Schober et al., 2007). Furthermore, the transmembrane receptor tyrosine kinases (RTKs) for epidermal and insulin growth factor (EGF and IGF) have critical roles in stimulating basement membrane proliferation (Barrandon and Green, 1987; Scholl et al., 2007; Zenz and Wagner, 2006). Overexpression of EGF can result in epidermal thickening (Atit et al., 2003), while deletion of Mig6, an antagonist of EGFR signaling, drives hyperproliferation of epidermal keratinocytes and consequently increases susceptibility to tumorgenesis (Ferby et al., 2006). Lrig1, a negative regulator of EGFR susceptibility, can repress keratinocyte proliferation in vitro (Jensen and Watt, 2006). β1 integrin and transforming growth factor α (TGFα) affect the proliferation of epidermal SCs positively, while TGFβ is a negative regulator that can induce G_1 cyclin-dependent kinase (CDK) inhibitors (Massague and Gomis, 2006) as well as apoptosis. Furthermore, c-Myc, which is a transcription factor and target of RTK and TGFβ (Oskarsson et al., 2006), as well as p63, a relative of tumor suppressor p53 (Truong et al., 2006), control epidermal proliferation.

Because skin is perpetually exposed to the environment, both wound repair and immunological response simultaneously have to proceed perfectly. Both processes are defined by well-regulated apoptosis and tissue degradation requiring a multitude of immune cells. Upon injury, initially neutrophils are attracted to inflammatory sites by interleukin (IL)-8, interferon gamma (IFN-γ) and C5a, then

monocytes, which differentiate into macrophages, and mast cells arrive from nearby tissue (Martin and Leibovich, 2005). Neutrophils and macrophages are very important because of their phagocytic function and the ability to secrete toxic mediators, such as ROS. Other phagocytosis-competent, myeloid cells are the Langerhans cells, which are elements of the adaptive immune system. Upon differentiation into antigen presenting cells (APC) they are responsible for antigen uptake, processing and presentation (Lippens et al., 2005).

Keratinocytes play an essential role in chemotaxis of leukocytes as well as of themselves, and in initiation of immune defense. In vitro they express Toll-like receptor (TLR)1-6, 9 and 10 mRNA, although TLR3-5 and 9 appear functional and induce immune-associated responses (Lebre et al., 2007). Expression of TLR5 and 9, controlled by TGF-α, result in upregulation of neutrophil-specific chemoattractant CXC ligand 8 (CXCL8)/ IL-8 and antimicrobial peptides (Selsted and Ouellette, 2005). Leukocyte-derived cytokines such as TNF-α, INF-γ, IL-1, IL-17 and IL-22 not only induce pro-inflammatory function of keratinocyte by inducing chemokine and cytokine expression thereby resulting in recruitment and activation of neutrophils, macrophages, dendritic cell (DC) precursors and T cells in the epidermis (Pastore et al., 2008), but are also known as activators of EGF family growth factors and EGFR (Valyi-Nagy et al., 1992).

1.2 Hair follicle morphogenesis and hair cycle

The formation of hair follicles is a complex process depending on a well regulated balance between cell proliferation, polarization, differentiation and apoptosis. Hair follicle morphogenesis occurs in waves from E14.5 to E 18.5 in mice, and starts with the formation of the hair placode, an invagination driven by mesenchymal-epithelial interaction (Fuchs and Nowak, 2008) and massive keratinocyte proliferation (Magerl et al., 2001). Induced by condensation of a specific set of fibroblasts the dermal papilla (DP) is formed, which drives further HF formation. Due to the highly proliferative and differentiating (matrix) cells at the leading front of the forming HF, the DP grows downwards into the dermis (Fig. 2). Moreover the inner root sheath (IRS) and the hair shaft (HS), originated from transiently amplifying matrix cells of the bulb, grow upwards. The outer root sheath (ORS) merge into the BM of the epidermis (Fuchs and Horsley, 2008; Fuchs and Nowak, 2008). At postnatal day

6 (P6) the bulb reaches the bottom of the dermis and the follicular maturation is fully completed. At that moment the transiently amplified matrix cells, located at the follicle base, continue dividing and produce progeny cells which later terminally differentiate to form the growing hair shaft and fibre.

Once mature, hair follicles enter the so called hair cycle this is a tightly regulated process of proliferation, regression and resting. When HFs enter the so called hair cycle the lower portion of them regress due to an apoptosis-driven degenerative phase called catagen. This phase is characterized by the end of HS differentiation and a transformation into the so called club, which remains anchored during the resting phase called telogen. The first resting phase in mice begins around P19 and is short, lasting 1 or 2 days, however the second lasts 2 weeks. A new round begins with transition from telogen to anagen, when some follicle stem cells in the bulge region start to proliferate rapidly and differentiate. The duration of anagen determines the length of HS, but generally the whole HF is long and straight in anagen compared to the other hair cycle phases (Alonso and Fuchs, 2006). Some important molecular regulators are EGF, FGF5, neurotrophins, p53 and TGFβ-family members (Foitzik et al., 2000; Hebert et al., 1994; Schmidt-Ullrich and Paus, 2005). Loss of EGFR in epidermis goes hand in hand with failure of hair growth. This was confirmed by grafting skin or skin cells from EGFR null mice to nude mice, which compromise effects of systemic disease (Hansen et al., 1997). Expression of dominant negative EGFR in epidermis and ORS results in hair follicle morphogenesis arrest (Murillas et al., 1995). However, loss of TGF-α, usually expressed in the IRS and ORS (Luetteke et al., 1993; Nixon et al., 1996), and EGF, expressed in the ORS in growing hair follicles (du Cros et al., 1992), shows a less severe phenotype of curly whiskers and less pronounced waviness of the first hair coat.

In WT mice during P7 and P14 EGF receptor is highly phosphorylated, whereas at P21, when hair follicles rest in the telogen phase the expression of EGFR is down regulated. At P35, when hair follicles remain in telogen the expression of EGFR is again down regulated. EGFR signaling is reactivated in anagen again. Generally EGF expression is increased a few days after birth and switched off when hair follicles enter catagen. Consecutive topical application of selective inhibitors of EGFR and ErbB2 in WT mice results in inhibition of hair growth and hair follicles are also stuck in anagen, confirming that EGFR/ErbB2 signaling is indispensable for hair growth (Mak and Chan, 2003).

With each new hair cycle, the resting phase expands, maybe due to the difficulty to achieve the threshold of stimulatory signals for telogen-to-anagen transition (Fuchs and Horsley, 2008).

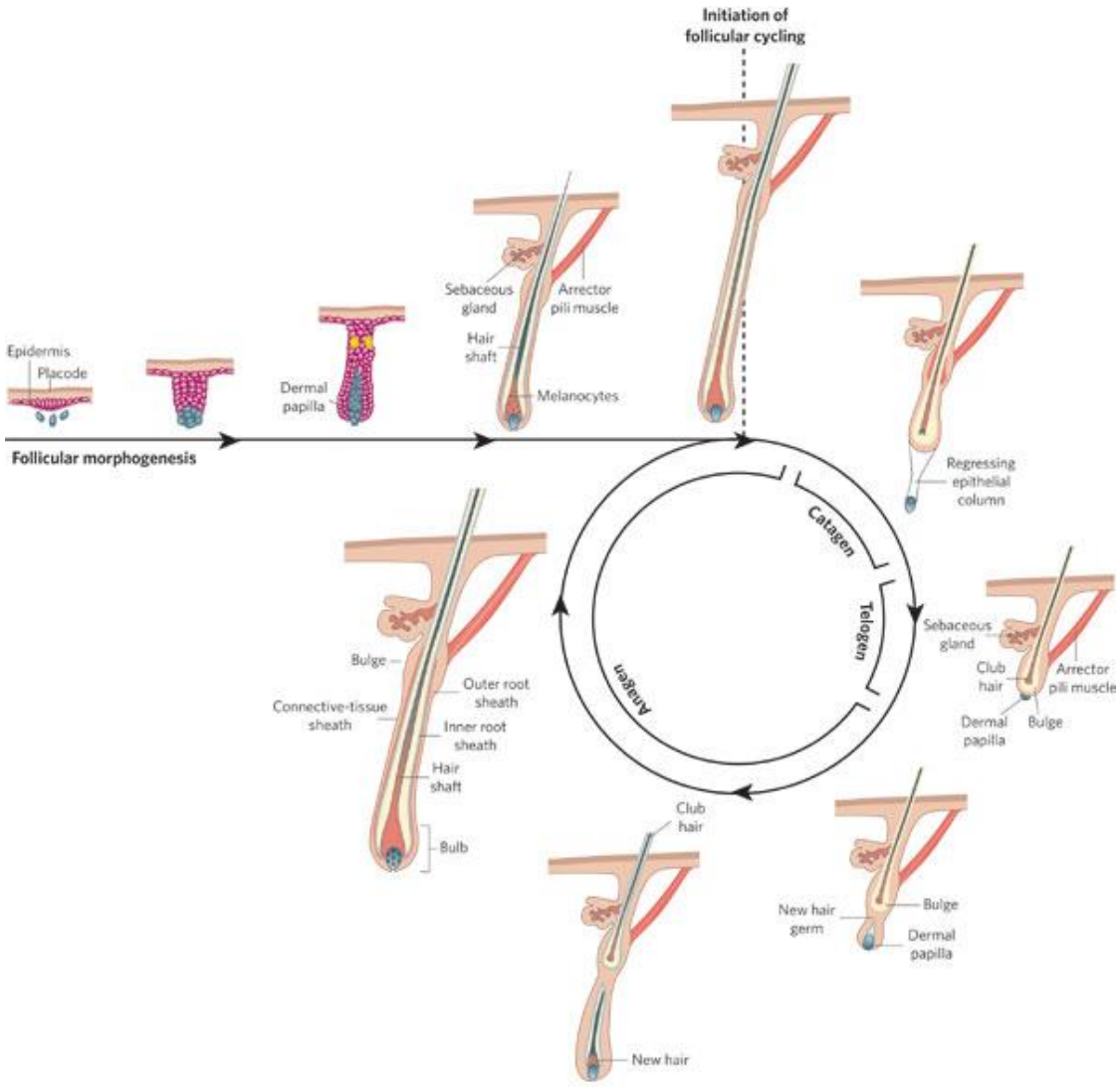


Figure 2 | Hair follicle morphogenesis and hair cycle. After termination of hair follicle morphogenesis, the hair cycle is initiated by a resting phase (telogen). It continuous with a destructive phase (catagen) and the stem cell activating regrowth phase (anagen) again (Fuchs, 2007).

1.3 Epidermal tumors

Due to the exposure to mechanical, chemical and UV damage the risk of tumor development in skin is very high. The ability for selfrenewal is maintained by epidermal stem cells, its transient amplified progenitors and the stem cells located in

the hair follicle. Stem cells are the main target cells for various types of epidermal tumors and due to the high division rate mutations can be manifested easily. Furthermore, more than one event is necessary to transform a normal stem cell into a cancer cell (Hahn et al., 1999). The most common epithelial tumors of the skin are basal cell carcinomas (BCCs) and squamous cell carcinomas (SCCs) in human, and SCCs and its precursor papillomas in mice. The most deadly form of skin cancer is the cutaneous melanoma. In the mouse SCC and papillomas originate from the IFE, while BCC is suggested to arise from undifferentiated follicle ORS due to mutations affecting the Hedgehog (SHH) signaling (Oro et al., 1997). Mutations in p53 and Ras seem to be the most important and powerful ones for the development of BCCs and SCCs. Although melanocytes, tightly regulated by keratinocytes, play an essential role in protecting the skin from UV radiation, they are the precursors of melanoma. In 50 to 70% of melanomas BRAF, one of the three *Raf* genes, displays a mutation of valine at position 600 (V600E). This alteration results in a constitutive activation of ERK signaling which again stimulates proliferation and survival, and provides tumor growth and function (Gray-Schopfer et al., 2007).

Genesis of epidermal tumors is not limited to genetic alteration in stem cells. The differentiating compartments can also be targets of alterations finally contributing to tumor formation. Transient amplifying cells, post-mitotic and terminally differentiated cells can undergo proliferation regulated by an oncogene. Moreover, differentiated epidermal cells affect proliferation of altered stem cells and consequently the creation of a tumor by enhancing or inhibiting signals (Owens and Watt, 2003). Many different genes are deregulated, including EGFR. Furthermore, molecules such as integrins, for instance, have bivalent functions. While α3β1 integrin reduces development of papillomas into SCCs (Owens and Watt, 2001), α6β4 integrin leads to an enhancement of papillomas, SCCs and metastasis. β1 integrin expression promotes an increased expression of IL-1α, which activates ERK/MAPK in keratinocytes. C-Myc causes vascular endothelial growth factor (VEGF) secretion by keratinocytes resulting in enhanced angiogenesis (Pelengaris et al., 1999). However, an arrest of clonal expansion is mediated by transforming growth factor-β (TGF-β) inhibiting epidermal proliferation (Akhurst et al., 1988).

2. The impact of inflammation on tumors development

Tumor development is a complex multistage process initiated by genetic alterations. Generally these mutations drive the transformation of normal cells into highly malignant ones. At least two genetic changes are required to receive a tumorigenic competence in rodent cells, while human cells are more difficult to receive a malignant phenotype or genotype (Hahn et al., 1999). The most important criterion a genetic mutation has to fit is that cells obtain growth advantages towards others. In detail, the genotype of cancer cells is defined by six essential alterations in cell physiology regulating malignant growth (Fig. 3): self-sufficiency in growth signals, insensitivity to growth-inhibitory (apoptotic) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. It is suggested that cells have to gain these alterations, but in random order, to become a cancer cell. However, a single genetic mutation can lead to more than one alteration. For instance, the loss of function mutation of the tumor suppressor gene p53 results both in tumor angiogenesis and resistance to apoptosis (Hanahan and Weinberg, 2000).

Since the last ten years scientists postulate that the amount of the six capacities a cell has to achieve to transform into a cancer cell is extended by a seventh capacity – inflammation. Many inflammatory diseases also trigger cancer development which leads to the suggestion of linkage between tumorgenesis and inflammation.

During the transformation, cells apparently have to develop the ability to grow in a chronically inflamed microenvironment, to escape immune recognition and to suppress immune reactivity (Cavallo et al., 2011). Both the intrinsic pathway (driven by genetic alteration) and extrinsic pathway (induced by inflammatory cells and mediators) mediate inflammation and neoplasia. Key regulators of the inflammatory response like cytokines, chemokines, lipid mediators, nitric oxide (NO), NFκB, HIF1α and STAT3 promote tumor development (Mantovani et al., 2008). Recent studies examined that NFκB controls macrophages polarization during tumor development (Hagemann et al., 2008). $STAT6^{-/-}$ mice harbor tumor associated macrophages (TAMs) displaying M1 phenotype leading to rejection of spontaneous carcinoma (Sinha et al., 2005). Activation of M1 phenotype by microbial products or INF-γ results in a high capacity of antigen presentation, secretion of IL-12 and IL-23 which

then provokes a type I T cell response. Furthermore, M1 macrophages display cytotoxic activity against tumor cells expressing NO, ROI and TNFα (Porta et al., 2009). STAT3, an immunosuppressor and responsible for evading the immune system, promotes the pro-carcinogenic IL-23 expression in tumour-associated macrophages (Porta et al., 2009).

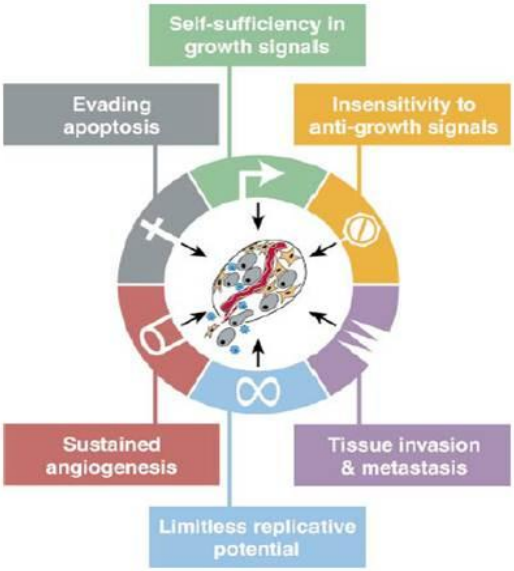


Figure 3 | Capabilities of Cancer. Cells have to gain certain skills during their transformation into a malignant cell (Hanahan and Weinberg, 2000).

Furthermore, the suppression and escape from immune responses is mediated by tumor-associated dendritic cell with immature phenotype and recruited myelomonocytic cells with an alternative M2 phenotype to downregulate the adaptive immune response against malignant cells. Tumor-associated macrophages (TAM), representing the majority of inflammatory cells, and myeloid-derived suppressor cells (MDSC) were shown to be attracted to tumors to promote its growth, dissemination and metastasis. The tumor-mediated M1-M2 switch of TAMs is an essential defense. While M1 macrophages are cytotoxic for cancer cells, expressing certain radicals and TNFα, the M2 phenotype is characterized by regulation of M1 inflammation, promotion of adaptive Th2 immunity, angiogenesis, tissue remodeling and repair (Gordon and Taylor, 2005). Furthermore, M2-polarized macrophages raise killing and encapsulation of parasites, support wound-healing and express tumor promoting functions (Porta et al., 2009). MDSC, expressing CD11b and Gr-1 in mice and known as T cell suppressors and cancer progression promoters, are regulated by colony stimulating factor-1 (CSF-1), IL-6, IL-10 and VEGF. IL-6 induces STAT3 resulting in a decrease of immune functions (Stewart and Trinchieri, 2009), IL-10 secreted by the

tumour induces the suppressive phenotype (Gallina et al., 2006) and VEGF, essential for the cross-talk between tumour and microenvironment, is directly involved in the recruitment of MDSC (Gabrilovich, 2004).

3. EGFR signaling pathways

The epidermal growth factor receptor (EGFR), also known as ErbB1, is the archetypal member of a family of four receptor tyrosine kinases which also includes ErbB2 (also known as HER2 or Neu), ErbB3 (HER3) and ErbB4 (HER4) (Gullick, 2001; Yarden and Sliwkowski, 2001). These receptors are responsible for mediating several cellular functions, including proliferation, survival, migration, and differentiation. An imbalance in both one and more of these receptors plays a central role in the etiology and progression of solid tumors (Earp et al., 1995; Jorissen et al., 2003).

The EGFR, a transmembrane protein, consists of three regions, the extracellular ligand binding region, the intracellular region with tyrosine kinase activity and a transmembrane region with a single hydrophobic anchor sequence, by which the receptor traverses the cell membrane (Voldborg et al., 1997). Dimerization and phosphorylation of the EGF receptor family are the initial and essential events of EGF-induced signal transduction. ErbB receptors are activated by a number of ligands (Fig. 4) referred to as EGF-related peptide growth factors (Riese and Stern, 1998). Each ligand has an EGF-like domain that is sufficient to confer binding specificity. These include EGF itself, amphiregulin (AR) and transforming growth factor-α (TGFα), which bind to ErbB1, and betacellulin (BTC), heparin-binding EGF (HB-EGF) and epiregulin (EPR), which exhibit dual specificity in that they bind both ErbB1 and ErbB4. Neuregulins (NRG) comprise the third ligand family. NRG-1 and NRG-2 both bind ErbB3 and ErbB4, whereas the more recent additions to the NRG family, NRG-3 (Zhang et al., 1997) and NRG-4 (Harari et al., 1999), bind ErbB4 but not ErbB3. Upon activation by their cognate ligands, ErbB receptors form dimers - either homodimers or heterodimers. Dimerization consequently stimulates the intrinsic tyrosine kinase activity of the receptors and triggers autophosphorylation of the five specific tyrosine residues within the cytoplasmatic domain removing an alternate substrate/ competitive inhibitor conformation (Voldborg et al., 1997). These

phosphorylated residues serve as docking sites for signaling molecules involved in the regulation of intracellular signaling cascades (Olayioye et al., 2000).

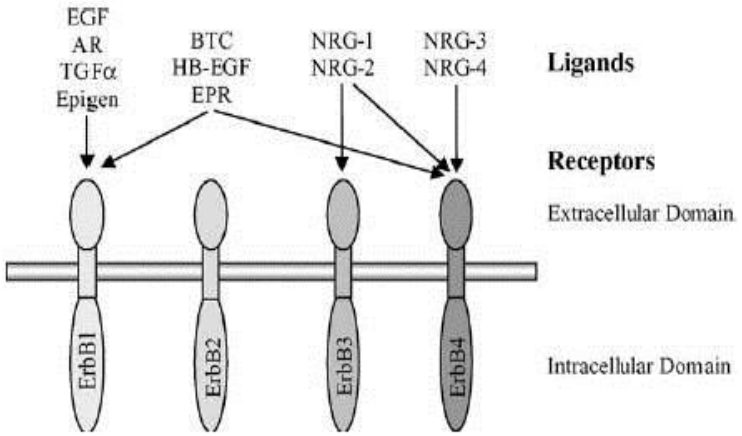


Figure 4 | Binding specificities of major EGF family ligands to the EGFR family receptors. There are four categories of ligands that bind ErbB family receptors. EGF, AR and TGFα bind ErbB1; BTC, HB-EGF and EPR bind ErbB1 and ErbB4; NRG-1 and NRG-2 bind ErbB3 and ErbB4; and NRG-3 and NRG-4 bind ErbB4 (Olayioye et al., 2000).

Generally phosphotyrosyl residues are recognized by intracellular proteins containing src homology 2 motifs (SH2). These proteins that interact directly or indirectly include enzymes such as PLC-γ1, GAP and syp phosphotyrosine phosphatase, as well as non-enzymatic adapter molecules such as the p85 subunit of phosphoinositol 3-kinase (PI3) and the src homology and collagen (Shc) protein (Voldborg et al., 1997).

The complexity of the ligands and induction of various downstream signaling pathways, including the Ras/MAPK, PI3K/AKT, and Jak/STAT pathway, ensure the specific regulation of cell proliferation, differentiation, migration and survival.

3.1 MAPK/Erk pathway

The mitogen-activated protein kinase pathway (MAPK), induced by EGFR, G-protein-coupled receptors and/or integrins and transduced by small GTPases such as Ras, is the major pathway mediating keratinocyte survival and proliferation (Kolch, 2000).

Upon ligand binding and dimerization, autophosphorylation of EGFR causes docking of the adaptor protein Shc of GDP/GTP-exchange factor son of sevenless (SOS). SOS induces a conformation change of Ras by inducing an exchange of the bound GDP with GTP. Ras itself owns a GTPase which hydrolyses the GTP to GDP

when signal transduction was performed. Ras can activate Raf-1 which in turn phosphorylates two serine residues of MAPK/ERK kinase (MEK1/2) and after recruitment to the membrane MEK1/2 is activated. MEK phosphorylates extracellular signal-regulated kinase (ERK1/2) at threonine and tyrosine residues in its active loop. ERK, a serine/threonine kinase, is a subgroup of mitogen-activated proteins (MAP) and upon activation it can phosphorylate over 80 substrates in the cytoplasm and the nucleus, for instance transcription factors like Ets, Elk, c-myc or c-fos (Orton et al., 2005). Transmission of signals is not only performed by ERK, but also via c-Jun NH_2-terminal kinase (JNK) and p38s. Generally p38 MAPKs regulate proliferation, differentiation, transformation and apoptosis (Weston and Davis, 2002).

3.2 PI3K/ Akt pathway

Another important pathway induced by EGFR is the phosphadityl inositol 3-kinase (PI3K)/ Akt pathway, which has an impact on cell survival via inhibition of Bad or forkhead transcription factors, which activate apoptosis related genes, and regulates chemotaxis and motility (Datta et al., 1997). Furthermore, nuclear factor κB (NFκB)-mediated expression of prosurvival genes as well as cell growth and indirect activation of mammalian target of rapamycin (mTOR) is modulated by Akt activity (Kane et al., 1999).

The initial step of activation of PI3K pathway is the recruitment of p85, the regulatory subunit of PI3K, to EGFR phosphotyrosine residues. Its catalytic subunit called p110 phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP_2) at the 3' position of the inositol ring, and generates PIP_3. PIP_3 in turn mediates the recruitment of phosphoinositide-dependent kinase 1 (PDK1) and PDK2 to the membrane, resulting in phosphorylation of two residues in the Akt kinase domain for full activation. Phosphate and tensin homolog (PTEN) attenuates the downstream signaling of PI3K by removing the 3' phosphate of PIP_3 again. Akt moves to cytoplama and nucleus to activate NFκB, human telomerase reverse transcriptase (hTERT), cyclin D1, hypoxia-inducible factor (HIF)-1α, eNOS and matrix metalloproteinases (MMP), and to inactivate Bad, caspase 9, p27 and p21 (Dillon et al., 2007).

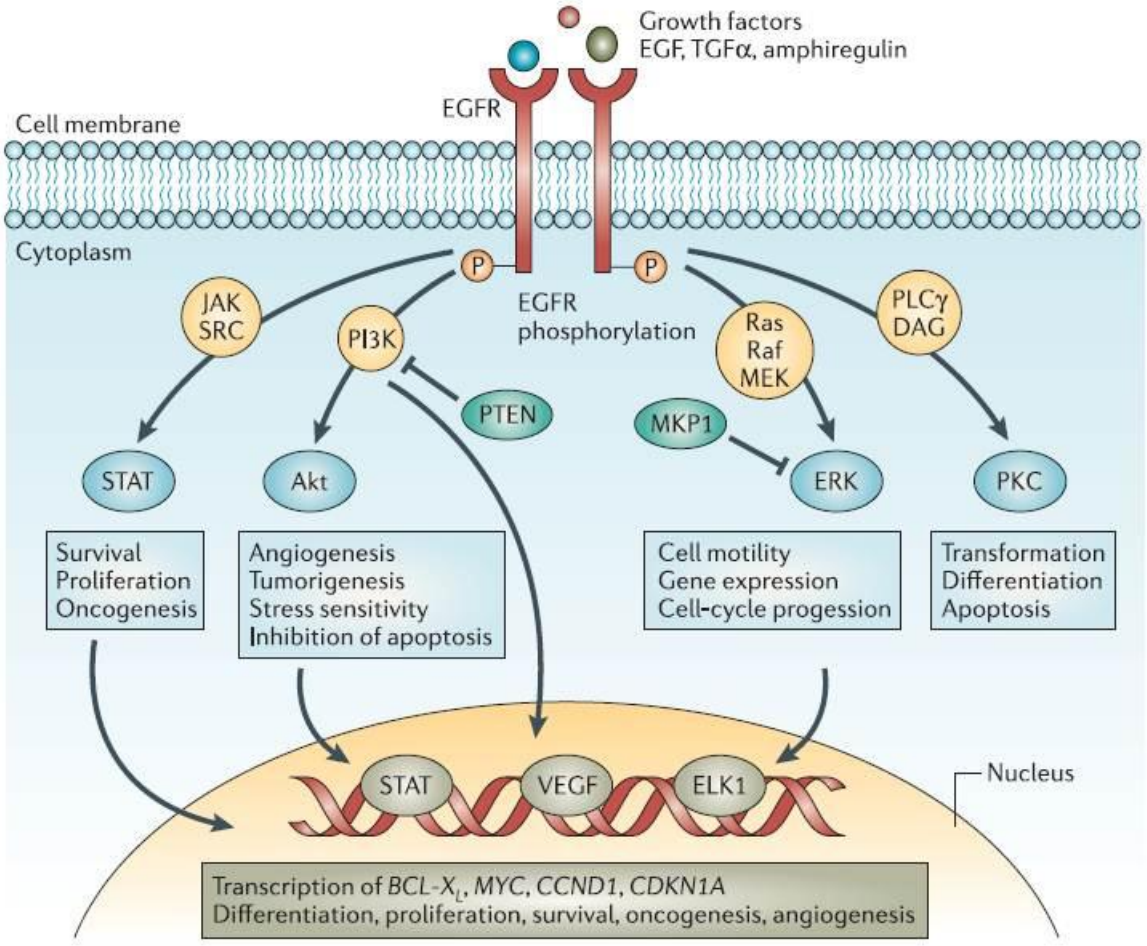


Figure 5 I The main downstream signaling pathways controlled by EGFR (Nyati et al., 2006).

3.3 STAT pathway

Activation of signal transducer and activator of transcription 1 (STAT1), STAT3, STAT5, is mediated by a janus kinas (JAK)-dependent and JAK-independent mechanism. The phosphorylated EGFR initiates dimerization of STATs (Fig. 4), which results in translocation into the nucleus, where gene transcription was activated. STAT3 activation increases cell proliferation in vitro and tumour growth rates in vivo (Andl et al., 2004).

3.4 EGFR in skin development

In order to maintain skin homeostasis and the controlled hair follicle growth as well as regression, proliferation and differentiation of epidermal cells must be strictly regulated. The EGFR is one of many receptors involved in maintaining skin development and homeostasis. In skin EGFR is expressed in the proliferation competent basal cells of the epidermis. However, expression is decreased as keratinocytes enter the program of terminal differentiation.

The first evidence of the EGF receptor's important role was in 1933, when Francis A.E. Crew postulated the mouse line *waved-1* (*wa-1*) which habours a point mutation in the gene encoding TGF-α, a ligand of EGFR (FAE, 1933). These animals develop a curly hairy coat. Two years later, the *wa-2* mice, containing a point mutation in the EGFR gene itself, were described and compared to wa-1 mice, wa-2 mice display a more marked phenotype (Luetteke et al., 1994). This confirms that mutations in the EGF receptor obviously affect the skin development more than its ligands.

Indeed, null mutations in individual ErbB loci are lethal. More specifically, depending upon the genetic background of the host, loss of EGFR (ErbB1) leads to embryonic or perinatal lethality within 3 weeks. These mice show abnormalities in multiple organs including the brain, skin, lung and gastrointestinal tract. $EGFR^{-/-}$ mice also show open eyelids at birth, and compromised epidermal and hair follicle differentiation (Miettinen et al., 1995; Sibilia et al., 1998; Sibilia and Wagner, 1995). Moreover $EGFR^{-/-}$ mice display a delay in hair follicle development and multiple hair shaft abnormalities, and show an aberrant wound healing response (Hansen et al., 1997). To circumvent the high lethality of $EGFR^{-/-}$ in the first month, a conditional knock-in mouse line ($hEGFR^{KI/KI}$) was generated, which live up to six months and express only very low levels of human EGFR in the skin. $hEGFR^{KI/KI}$ displays curly whiskers, and an altered morphology, and distribution of hair follicles. Similar to other mice harboring mutations in the EGFR pathway, $hEGFR^{KI}$ displays a strong infiltration, mainly macrophages, lymphocytes, neutrophils and multinucleated giant cells, with most likely leads to the loss of most hair follicles over time (Sibilia et al., 2003).

In summary, EGFR and its ligands play an important role in skin development and homeostasis. EGFR orchestrates proliferation and differentiation of interfollicular

and follicular epidermal cells. Furthermore, EGFR signaling pathway influences hair follicle morphogenesis and cycling.

3.5 EGFR in skin cancer

EGFR signaling pathways are very important for keratinocytes cell fate, because a modification of those pathways results in mesenchymal-epithelium alterations. Both EGFR overexpression and mutations have been observed in a wide variety of malignancies (colorectal, breast, NSCLC, pancreatic, gastric), which has stimulated interest in EGFR as a target for anticancer therapies. Epidermal tumors frequently display EGFR overexpression (Hunts et al., 1985). In contrast, loss of function of EGFR inhibits the development of skin tumors, confirmed by grafting EGFR-null keratinocytes expressing *v-ras*^{Ha} which results in size reduction of papillomas (Dlugosz et al., 1997). Moreover K5-SOS-F mice, expressing human SOS in the epidermis and ORS, develop spontaneous papillomas. Tumorgenic response is reduced in K5-SOS-F transgenic EGFR-null mice, suggesting that EGFR is essential for the development of skin tumors (Sibilia et al., 2000). A similar effect is proven when K5-SOS-F mice are crossed to wave-2 mice (Luetteke et al., 1994). In addition, EGFR is essential for maintaining the proliferative population in basal cell compartments of papillomas (Hansen et al., 2000). In addition, it was shown that EGFR is a survival factor for epidermal tumor cells (Sibilia et al., 2000). Furthermore, EGFR acts as a regulator of VEGF and Ang-1 expression accentuating the fundamental role in cancerogenesis (Casanova et al., 2002). The interaction of EGFR and VEGF has been confirmed by deletion of both one VEGF allele as well as both VEGF alleles in EGFR$^{wa2/\ wa2}$ and EGFR$^{\Delta epER}$ background. In the absence of EGFR and VEGF tumor development was inhibited in EGFR$^{wa2/\ wa2}$, and K5-SOS-dependent tumor size was severely reduced in the epidermis suggesting that EGFR expression is sufficient (Lichtenberger et al., 2010).

3.6 Interplay between immune system and EGFR signaling

Dermatological toxicities have been described in patient treated with EGFR inhibitors (EGFRIs) which is a common therapy against various malignancies. Those

dermatologic alterations are papulopustular rash, dry skin, itching, and hair and periungual alterations (Lacouture, 2006).

There is increasing evidence that the EGF receptor and its downstream targets are involved in immune responses of the skin. It was shown that EGFR inhibition can lead to the recruitment and activation of immune cells because its activation is responsible for down regulation of TNF-α- or INF-γ-induced CCL5/RANTES (Regulated upon Activation, Normal T-cell Expressed, and Secreted) and CCL2/MCP-1 (monocyte chemotactic protein-1) expression in human keratinocytes. Neutrophils, monocytes/macrophages and DC precursors as well as T cell immigration to skin lesions is impaired upon the EGFR activation *in vivo*. *In vitro* experiments in epidermal keratinocytes show a severely increased level of CCL2, CCL5 and CCL10, and decreased level of CCL8 when EGFR or ERK is inhibited (Pastore et al., 2005). Furthermore, ERK or EGFR inhibition, support the function of antigen-presenting cells, such as Langerhans cells, by enhancing their expression of IL-1β (Fujita et al., 2007).

Furthermore, upregulation of EGFR and its ligands is characteristic for chronic inflammatory skin disorders such as psoriasis. Suprabasal expression of amphiregulin causes severe psoriasis-like hyperplasia as well as dermal and epidermal infiltration of neutrophils and lymphocytes (Cook et al., 1997). Additionally, deficiency of epiregulin results in chronic dermatitis which is correlated with increased expression of the pro-inflammatory cytokine IL-18 by keratinocytes (Shirasawa et al., 2004).

The distinct function of EGFR, its ligands and downstream targets in regulation and interference of the immune response in skin is still an unknown field in science.

4. Tumor model

Chemical induction of tumors in mouse skin has been used to study mechanisms of epithelial carcinogenesis and evaluate modifying factor. The model of the multi-stage chemical carcinogenesis (Fig. 6) represents one of the best established *in vivo* models for the study of the sequential and stepwise development of tumors. It can be used to evaluate both novel skin cancer prevention strategies, the impact of genetic background and genetic manipulation on tumor initiation,

promotion and progression. Cell proliferation and hyperplasia, two characteristics of multistep evolution of cancer, influence the development of the two-stage protocol for mouse skin tumorgenesis (Kemp, 2005). While in other carcinogenesis protocols tumor development does not require treatment with promoting agents , in two-stage skin carcinogenesis the promotion via a tumor-promoting agent, for instance 12-O-tetradecanoylphorbol (TPA), plays an essential role (Abel et al., 2009).

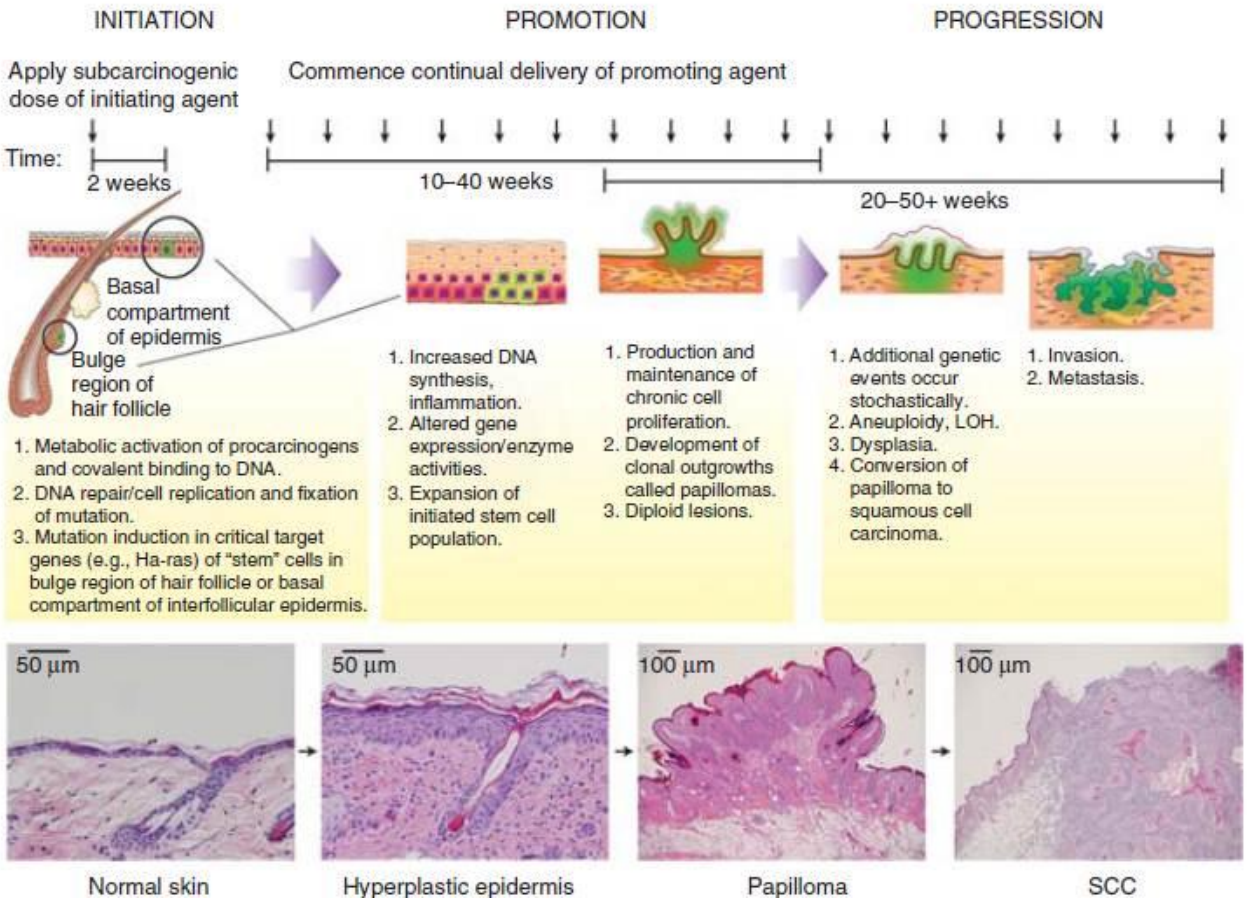


Figure 6 | Two-stage model of skin carcinogenesis in mice. Initiation of mutations in the target genes in keratinocyte stem cells is induced by a single topical application of a sub-carcinogenic dose of a mutagenic agent. During this stage, mutation in critical target genes of stem cells in the bulge region of the hair follicle or basal compartments of interfollicular epidermis is essential. Two weeks after initiation a promoting agent is regularly and topically applied. "Promotion" is characterized by an increased DNA synthesis and inflammation, and an expansion of the initiated stem cell population. Progression, the last stage of this model, is defined by additional genetic events such as aneuploidy, loss of heterozygosity (LOH), dysplasia, conversion of papilloma to squamous cell carcinoma, invasion and finally metastasis (Abel et al., 2009).

Tumor initiation, an irreversible step, is applied by a single sub-carcinogenic dose of a carcinogen such as 7,12-dimethylbenz[a]anthracene (DMBA). Keratinocyte stem cells, localized in the interfollicular epidermis and the bulge region, are believed to be the primary cellular target of the initiation stage. The single topical application of DMBA induces metabolic activation of procarcinogens and covalent binding to the DNA, inducing for instance an A to T transversion in codon 61 of the Hras1 gene (Brown et al., 1990). Mutations in Hras1 are observed in the majority of papillomas (Balmain et al., 1984). The fixation and propagation of mutations are enabled by DNA repair and cell replication. Tumor promotion is induced by the repeated topical application of chemical agents or wounding (Kemp, 2005), which stimulates cell signaling, induces production and release of growth factors, generates oxidative stress, proliferation of basal keratinocytes, increased DNA synthesis and an inflammatory cell infiltration leading to epidermal hyperplasia (DiGiovanni, 1992; Kemp, 2005). The development of papillomas was described during promotion, in a time span of 10 to 40 weeks after initiation. The success of two-stage skin carcinogenesis in mice is known to be highly dependent on genetic background (DiGiovanni, 1992). Metabolizing the initiating agents, formation and removal of covalent DNA-adducts do not significantly differ, thus, the susceptibility lies in the response to the tumor promotion (Abel et al., 2009). They may progress to invasive squamous cell carcinomas (SCC). SCCs are downward invading lesions that are highly vascularized. Numerous gene expression changes are present, including those associated with epithelial-mesenchymal transition (EMT) (Caulin et al., 1993; Navarro et al., 1991). An advantage of this model is that tumor development can conveniently be monitored visually throughout the life span of the mouse and that efficacy of chemopreventive agents or the effect of dietary manipulation can be determined (DiGiovanni, 1991).

5. EGFR mutant mouse strains used in this study

To investigate the role of EGFR in skin physiology and pathology different EGFR mutant mouse strains were used: $EGFR^{-/-}$ mice show strain-dependent phenotypes with fatal defects in neuronal and epithelial tissues and die at different stages of embryonic and early postnatal development dependent on their genetic

background (Miettinen et al., 1995; Sibilia et al., 2007; Sibilia et al., 1998; Sibilia and Wagner, 1995; Threadgill et al., 1995).

EGFR$^{wa2/wa2}$ mice contain a T-to-G transversion mutation in the sequences encoding the tyrosine kinase domain on chromosome 11, resulting in a substitution of a glycine for a highly conserved valine at position 743 in the third kinase subdomain. The ability of wa2 EGFR to phosphorylate an exogenous substrate is reduced by > 90% compared with that of wild-type receptor (Luetteke et al., 1994).

The conditional EGFR mice (EGFR$^{fl/fl}$), which were generated in the laboratory of Dr. Sibilia (Natarajan et al., 2007) carried either floxed or flirt alleles (Fig. 7). The EGFR flirt allele is a floxed allele containing a Neo-cassette. Both alleles behave the same and result in a delta (Δ) allele lacking the promoter and exon 1 of EGFR upon Cre-mediated recombination. These EGFR$^{fl/fl}$ mice were crossed to K5-Cre (Tarutani et al., 1997) and K5-CreER^{T} (Indra et al., 1999) transgenic lines resulting in mice , where the receptor is constitutively deleted already during embryogenesis (EGFR$^{\Delta ep}$), and mice, where EGFR deletion can be induced postnatally by the injection of tamoxifen (EGFR$^{\Delta epER}$).

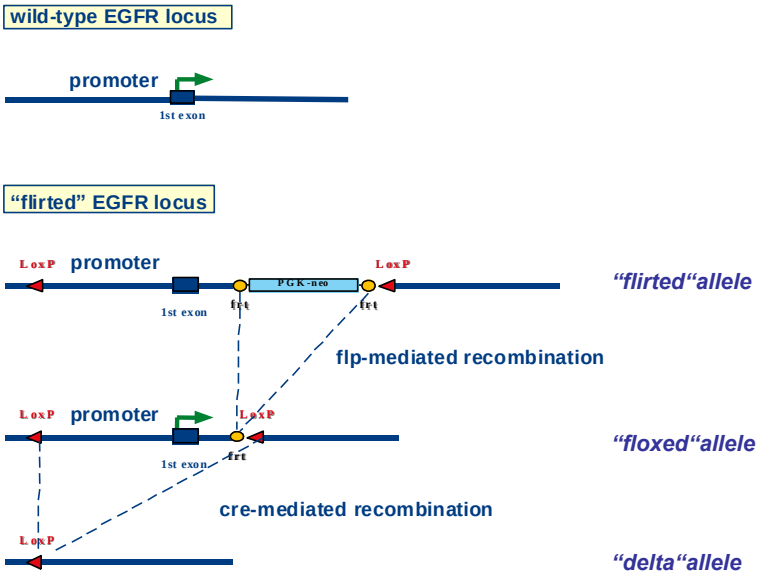


Figure 7 I Generation of conditional alleles of EGFR. The EGFR flirt allele is a floxed allele containing a Neo-cassette. It behaves like the EGFR floxed allele. Upon Cre-mediated recombination exon 1, which is flanked by lox-P sites, is deleted.

Results

1. EGFR$^{\Delta ep}$ mice show a delay in hair cycle and do not enter catagen

Several studies in EGFR mutant mice have shown that EGFR signaling plays an important role during hair follicle morphogenesis and HF cycling (Mak and Chan, 2003). However, as EGFR k.o. mice die shortly after birth, a detailed analysis could not be performed. Therefore, I addressed the effect of epidermis-specific EGFR deletion in EGFR$^{\Delta ep}$ mice at different phases of the hair cycle. Histological analysis of skin sections revealed that HF length is different in EGFR$^{\Delta ep}$. While at P8 HFs of EGFR$^{fl/fl}$ mice obtain 650µm, HFs of mice lacking EGFR in epidermis reaches only half of that size and at P10, when HF are fully developed, HF from EGFR$^{\Delta ep}$ mice only reach 77,43% of the length of wt controls (Fig. 8A). In addition, the analysis demonstrates that the lack of epidermal EGFR prevents HF from entering catagen, and, thus reveals a delay in HF cycling. At P20 the EGFR$^{\Delta ep}$ HFs still arrest in anagen, while in wild-type skin degradation of HF is already completed and they rest in telogen (Fig. 8A).

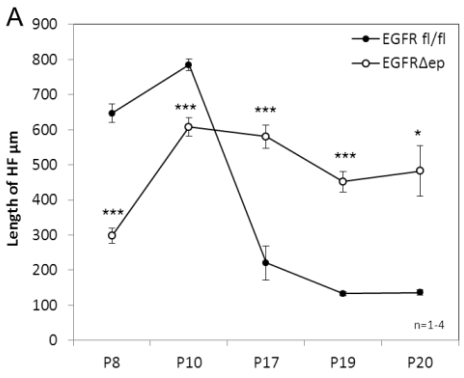
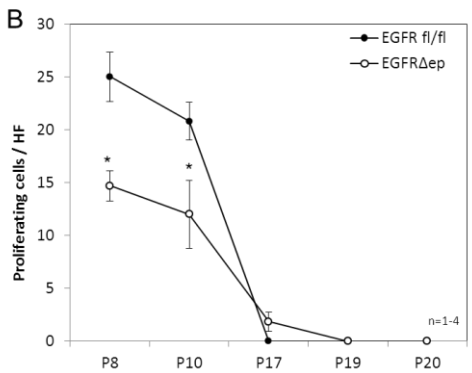
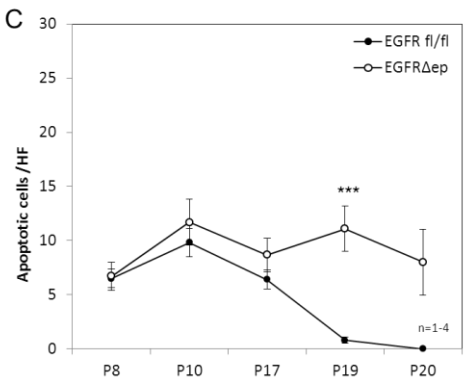
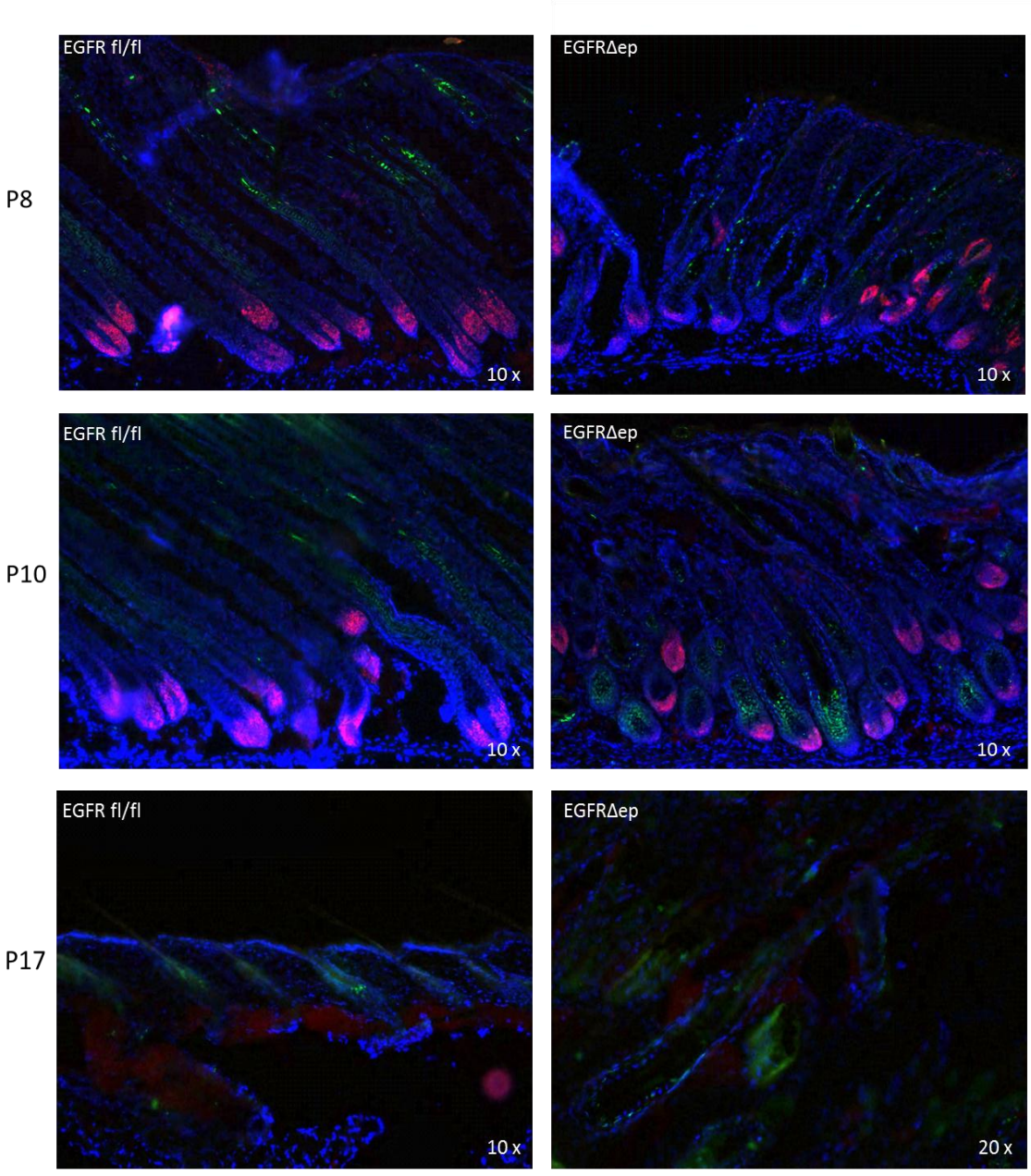


Figure 8 I Histological analysis of back skin of EGFR$^{fl/fl}$ and EGFR$^{\Delta ep}$ mice at different days after birth (P8, P10, P17, P19, P20; A-C). Analysis of hair follicle (HF) length in back skin labeled with DAPI (A). Quantitative analysis of proliferating (B) and apoptotic cells per HF (C). Data represent mean ± SEM. *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005





Moreover, double staining for proliferating and apoptotic cells in hair follicles was performed (Fig. 9). Interestingly, the number of proliferating cells in EGFR$^{\Delta ep}$ HF is significantly lower at all time-points compared to EGFR$^{fl/fl}$ (Fig. 8B, 9) which might explain the reduced HF length in EGFR$^{\Delta ep}$ skin (Fig. 8A). Moreover, whereas the number of apoptotic cells in wild-type HF increases at P10 and declines continuously in accordance with A/C transition and entry of the resting phase/ telogen, the number remains nearly the same in EGFR$^{\Delta ep}$ (Fig. 8C, 9). These results demonstrate that EGFR is necessary for proper progress of HF cycle due to regulation of proliferation and apoptosis.



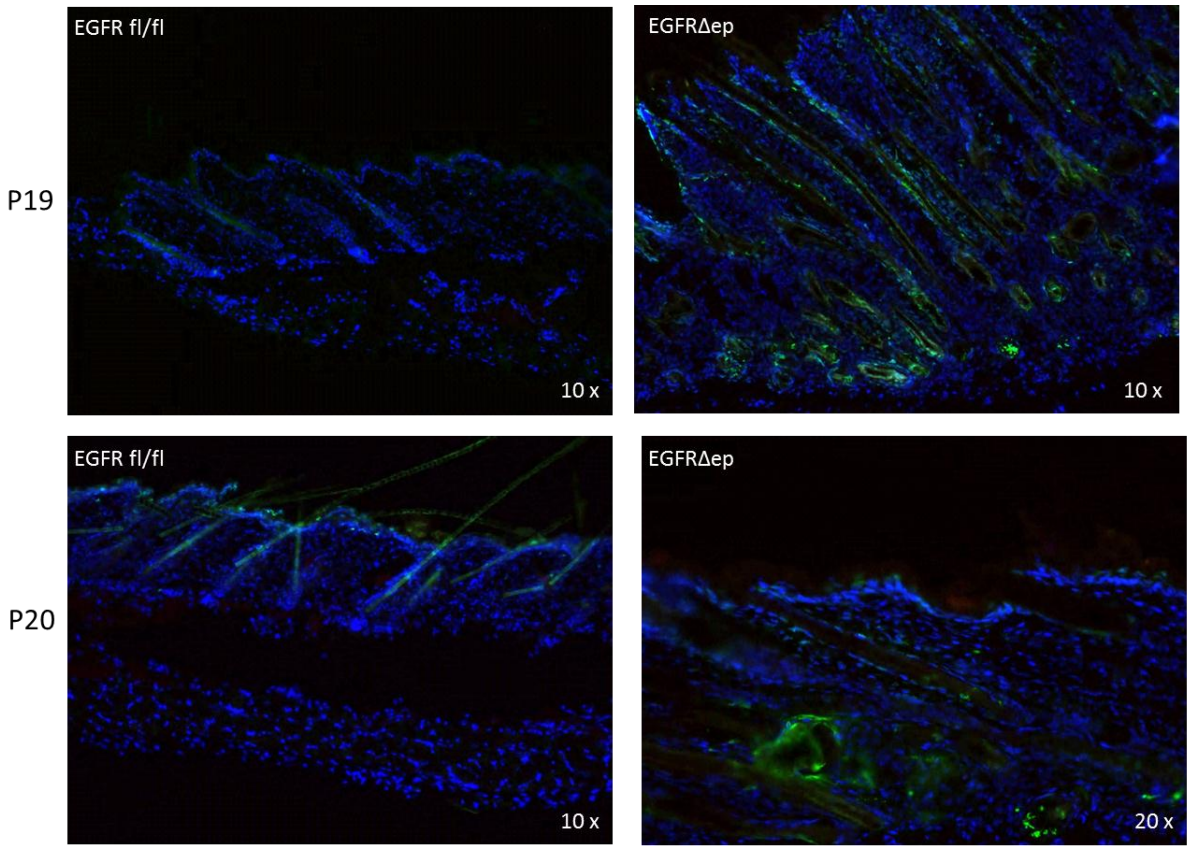


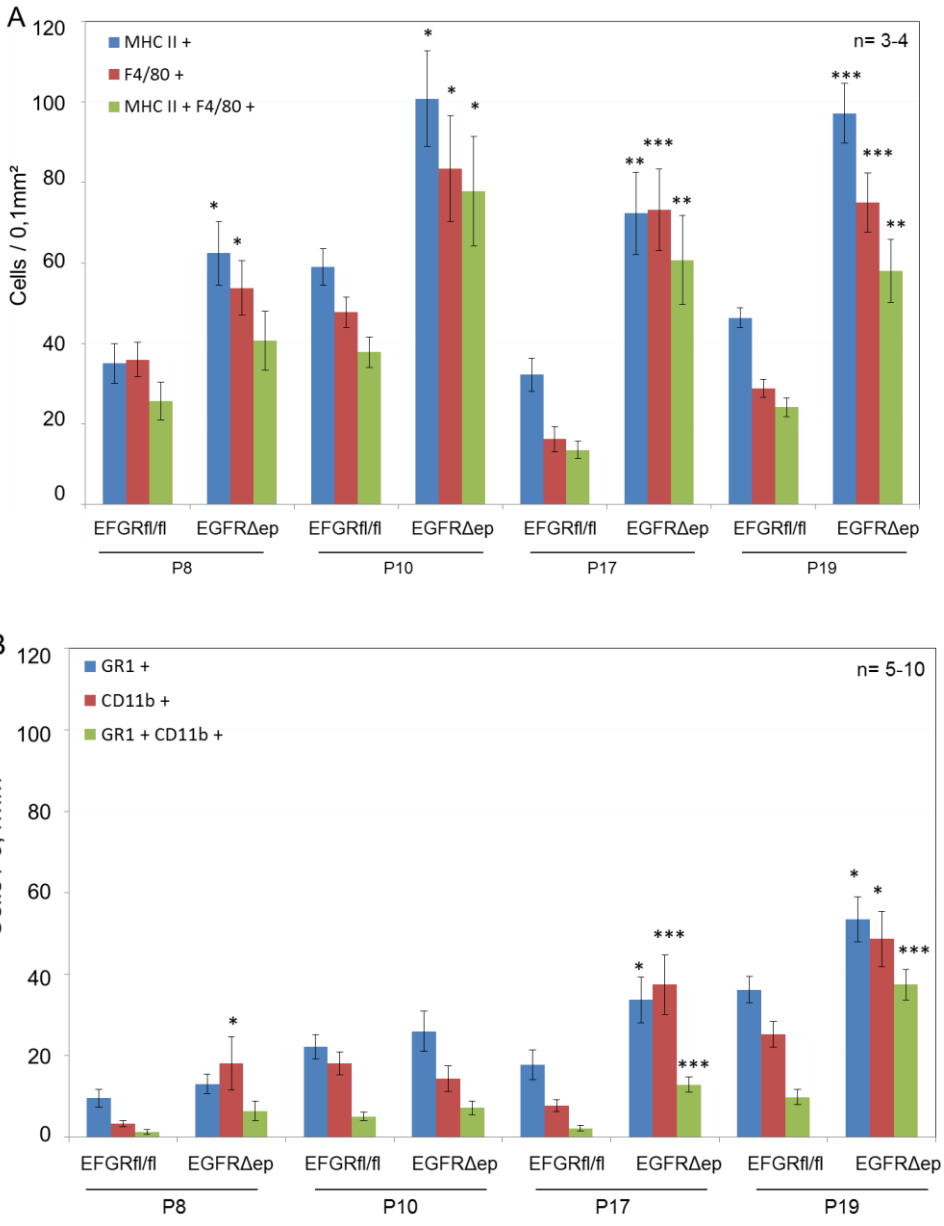
Figure 9 | Skin sections of EGFR$^{fl/fl}$ and EGFR$^{\Delta ep}$ mice at different days after birth (P8, P10, P17, P19, P20). Quantification of apoptotic cells, using ApopTag Detection Kit, and of proliferating cells, using Ki67 staining.

2. EGFR$^{\Delta ep}$ mice display strong infiltration of immune cells

The alternating growth and degradation phases of HFs during the hair cycle are accompanied by attraction of different immune cells (Ito, 2010; Ito et al., 2008). Histological analysis of skin sections from mice with mutations in the EGFR signaling pathway demonstrated increased skin inflammation. To address if the inflammation found in EGFR mutant skin is due to alterations of HF morphogenesis or cycling histological analysis was performed. Back skin samples of EGFR$^{\Delta ep}$ and EGFR$^{fl/fl}$ mice were isolated at different days after birth (P8, P10, P17, P19) to exhibit immune cell infiltration in a time dependent manner. O.C.T. embedded sections were double-stained with FITC and PE labeled antibodies detecting proteins like MHC class II and F4/80, GR1, CD11b, CD11c, CD3ε and langerin. Quantitative analysis revealed a significant increase of cells expressing MHC II and/or F4/80, which are macrophages respectively, in mice lacking EGFR in epidermis at all time-points (Fig. 10A).

Moreover the number of cells expressing GR1 and/or CD11b, which are myeloid granulocytes, monocytes and early hematopoietic dendritic progenitor cells respectively, was always higher in EGFR$^{\Delta ep}$ skin, especially at late stages (P17, P19) (Fig. 10B). Accordingly, also the infiltration of CD11c^{+} and/or MHC II^{+} cells, representing dendritic cells, was always increased in EGFR$^{\Delta ep}$ mice compared to their littermate controls with a peak at postnatal day 10 (Fig. 10C). No significant difference was detected in CD3ε^{+} cells, which are T-cells respectively, and langerin^{+} cells infiltration, except that at P17 Langerhans cells and at P19 CD3ε^{+} cells were significantly increased in EGFR$^{\Delta ep}$ mice compared to the control (Fig. 10D).

Taken together these results show a strong infiltration of several immune cells in EGFR$^{\Delta ep}$ mice during different stages of the hair follicle cycle.



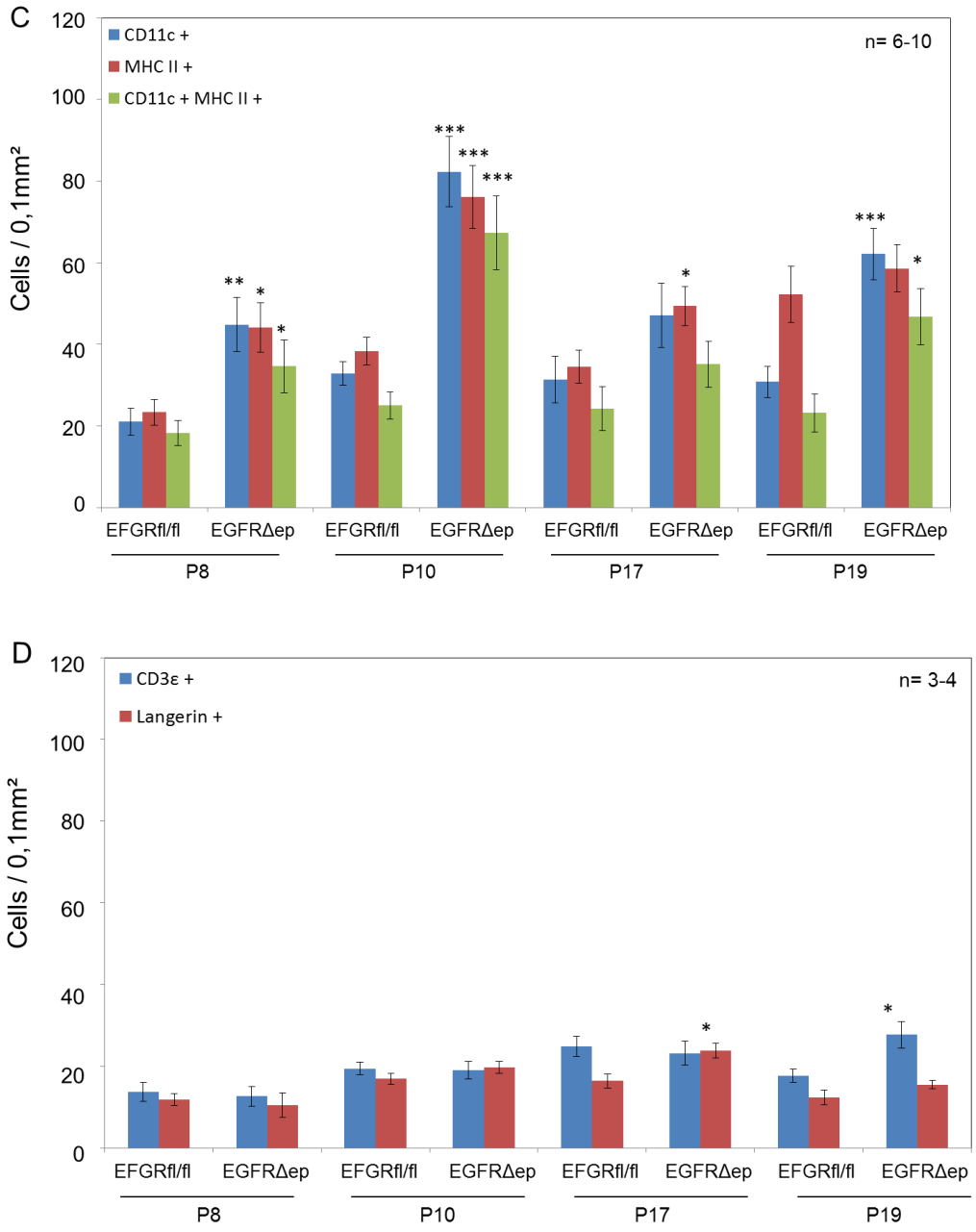


Figure 10 | Quantitative analysis of immune cells in EGFR$^{\Delta ep}$ and EGFR$^{fl/fl}$ skin (A-D). MHC II^{+} and/or F4/80^{+} cells are significantly increased during the whole hair cycle (A). GR1^{+} and/or CD11b^{+} cell infiltration is also increased in EGFR$^{\Delta ep}$ mice compared to EGFR$^{fl/fl}$ (B). Both the number of MHC II^{+} and/or CD11c^{+} cells is elevated in mice lacking EGFR (C). Numbers of T-cells and LCs are similar (D). Data represent mean ± SEM. *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005

3. Postnatal deletion of EGFR does not affect the number of epidermal Langerhans cells

In vitro inhibition of ERK, a downstream target of EGFR, results in enhanced expression of stimulatory and adhesion molecules by Langerhans cells, thereby improving their function (Fujita et al., 2007). To investigate if EGFR deletion affects the number of LCs, ear sheets from both $EGFR^{\Delta ep}$ and $EGFR^{\Delta epER}$ mice and the respective littermate controls were stained for the surface proteins MHC II and langerin.

While in $EGFR^{\Delta ep}$ mice Langerhans cells seem to be decreased in number in epidermal ear sheets compared to $EGFR^{fl/fl}$ mice, no difference could be detected between $EGFR^{\Delta epER}$ and $EGFR^{fl/fl}$ mice (Fig. 11). A possible reason may be that deletion of EGFR is insufficient in these mice so that no difference can be seen in the number of Langerhans cells or that inflammatory phenotype is milder in mice where EGFR is deleted after birth compared to mice where EGFR is already deleted during embryogenesis. Furthermore, despite the fact that *in vitro* inhibition of EGFR improves Langerhans cell function (Fujita et al., 2007), the reduction of Langerhans cells in $EGFR^{\Delta ep}$ mice suggests, that EGFR may be important for Langerhans cell regulation or recruitment.

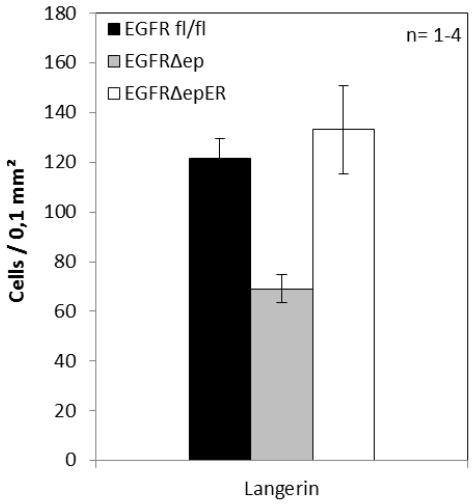


Figure 11 | Quantification of LCs in $EGFR^{\Delta ep}$ and $EGFR^{\Delta epER}$ mice. Histological analysis of Langerhans cells suggest a decrease in $EGFR^{\Delta ep}$ ears, while Langerhans cells infiltration seems to be not affected in $EGFR^{\Delta epER}$ ears compared to respective littermate controls. Data represent mean ± SEM.

4. Lack of epidermal EGFR affects TPA-dependent hyperplasia

12-*O*-Tetradecanoylphorbol-13-acetate (TPA) is a diester of phorbol and a potent tumour promoter often employed to activate the signal transduction enzyme protein kinase C (PKC) (Niedel et al., 1983). Topical application of TPA on the mouse skin is a well-known model for an induction of oxidative stress, ROS production, cutaneous inflammation and subsequently occurring hyperplasia. The inflammatory process is characterized by a recruitment of inflammatory cells such as neutrophils by chemotactic factors to inflammatory regions and edema formation (Nakamura et al., 1998).

As previously described conditional EGFR mice harbouring a Cre-transgene were treated with tamoxifen (TX) to induce EGFR deletion in the skin. By Western Blot the loss of EGFR protein of $EGFR^{fl/fl}$ K5-CreER mice ($EGFR^{\Delta epER}$) in the epidermis upon TX treatment was proven (Fig. 12).

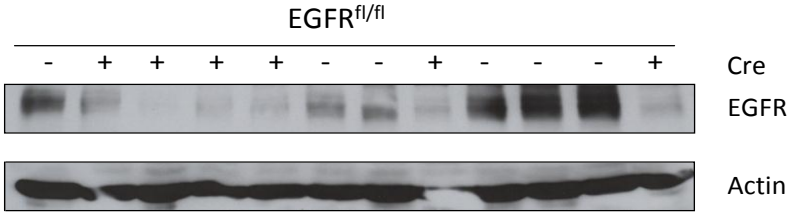


Figure 12 | EGFR is efficiently deleted in $EGFR^{\Delta epER}$ epidermis upon TX treatment. Western blot showing EGFR expression. Actin was used as a loading control.

Subsequently the back skin was shaved and treated with the chemical carcinogen 12-tetradecanoyl-phorbol-13-acetate (10µg TPA/ 200µl acetone) for the following 3 days. Skin samples were prepared to investigate the skin morphology by hematoxylin/eosin staining (H&E).

Histological analysis by H&E staining points out that the epidermis is thicker after the TPA treatment, however TPA-treated mutant mice – $EGFR^{\Delta epER}$ – display a less thick epidermis as compared with the treated controls (Fig. 13A). The general increase of epithelial cells induced by the tumour promoting TPA treatment seems to be prevented in keratinocytes lacking EGFR, which could underline the importance of EGFR as a survival factor under stressful conditions.

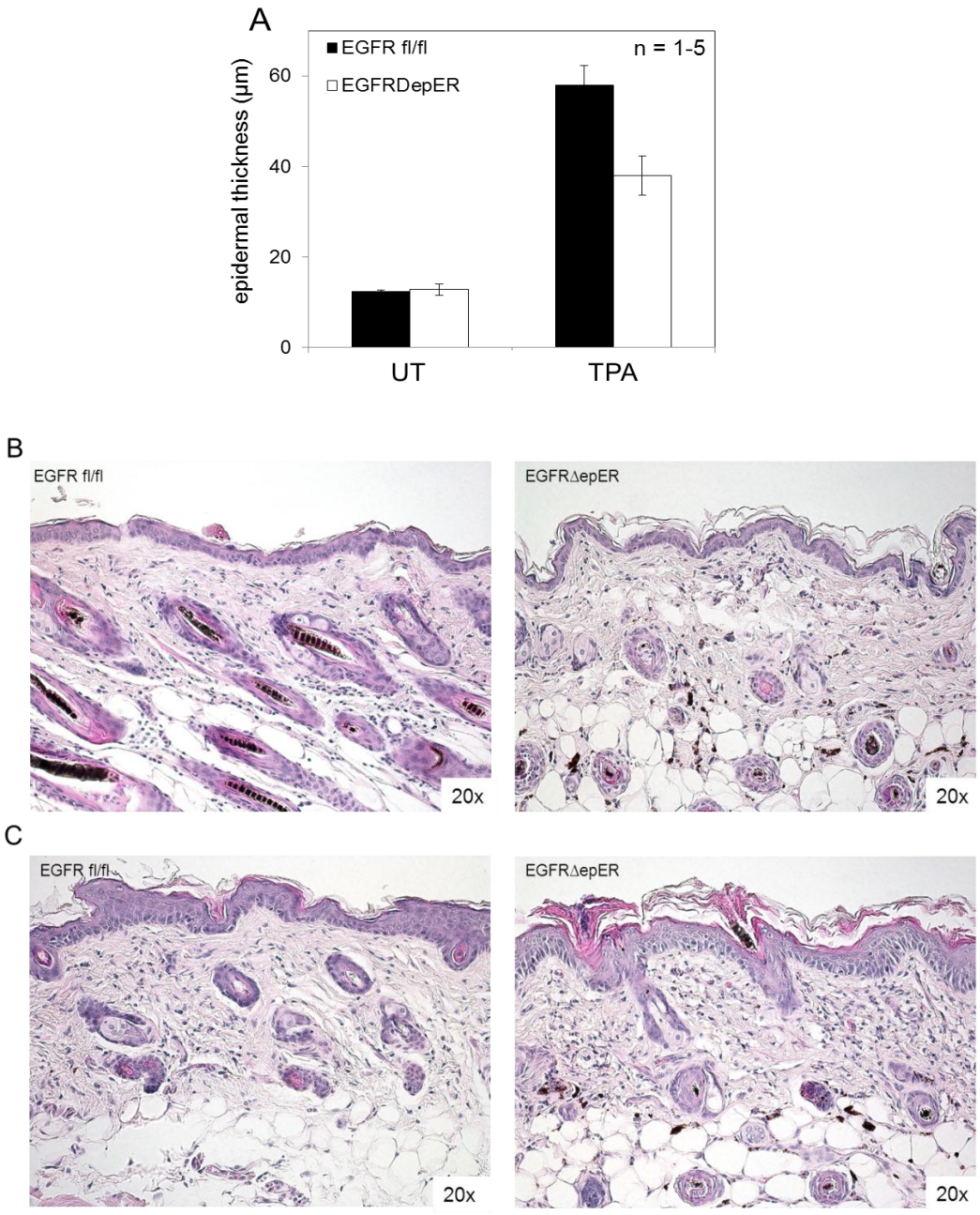


Figure 13 | TPA-mediated increase of epidermal thickness (A-C). Comparison of epidermal thickness of TPA-treated (C) and untreated (B) EGFR$^{fl/fl}$ and EGFR$^{\Delta epER}$ skin, H&E staining.

5. EGFR deficiency reduces TPA-dependent epidermal apoptosis and cell proliferation

Furthermore, skin sections from TPA-treated mice were analysed with TUNEL method, utilizing an anti-digoxigenin antibody conjugated to a Fluorescein reporter molecule, quantifying all apoptotic cells in the epidermis. However during

differentiation keratinocytes extrude the nucleus and are TUNEL positive. TPA-mediated stress induces apoptosis in both $EGFR^{fl/fl}$ and $EGFR^{\Delta epER}$ mice, as expected, but interestingly the number of apoptotic cells was significantly decreased in $EGFR^{\Delta epER}$ mice compared to $EGFR^{fl/fl}$ mice (Fig.14A). They also showed a lower, albeit non-significant, number of $Ki67^{+}$ cells upon TPA treatment (Fig.14B). This impaired proliferation suggests the reduced epidermal thickness upon TPA treatment. However, the reduction in apoptotic cells was rather unexpected as it was demonstrated that EGFR is a survival factor for epidermal cells in skin tumors (Sibilia et al., 2000).

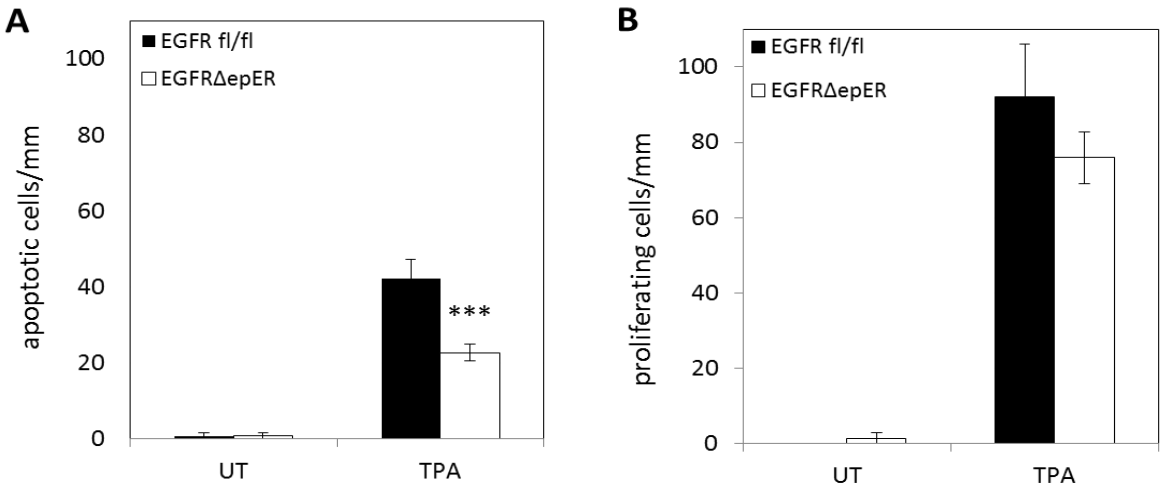


Figure 14 | Reduced apoptosis after TPA treatment in $EGFR^{\Delta epER}$ mice (A) and $Ki67^{+}$ cells (B). Quantitative analysis of apoptotic cells in skin sections isolated from untreated (UT; n= 2) and TPA-treated (TPA) mice (n= 4/5). Quantification of $Ki67^{+}$ cells in skin sections isolated from untreated (UT; n=2) and TPA-treated (TPA) mice (n=6). Data represent mean ± SEM. *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005

6. Lack of EGFR affects TPA-mediated activation of various signaling pathways

Next, molecular changes involved in the signaling pathway downstream of EGFR in TPA treated keratinocytes were investigated. Therefore epidermal cells were isolated from $EGFR^{\Delta epER}$ and $EGFR^{fl/fl}$ mice, processed to protein lysates and analyzed by Western Blot. Since no difference in EGFR protein level was detected in these total epidermis lysates (data not shown), keratinocytes of $EGFR^{fl/fl}$ and

EGFR$^{\Delta ep}$ mice were isolated and cultured until they reached 80% confluence. Cells were starved for 24hr and incubated either with 100ng/ml TPA or 20ng/ml EGF and 100ng/ml TPA for 5 min. Western Blot analysis revealed that EGFR was efficiently deleted in EGFR$^{\Delta ep}$ epidermal cells (Fig. 15). While TPA treatment has no impact on AKT, pNFκB and p-p38 activation in wild type keratinocytes, pERK1/2 protein level is increased upon TPA treatment. Moreover, both TPA-mediated AKT and ERK1/2 activation is increased compared to wt, but TPA-mediated activation of NFκB and p38 is independent of EGFR signaling in EGFR$^{\Delta ep}$ epidermal cells. The strong TPA-mediated activation of AKT as well as ERK1/2 in keratinocytes lacking EGFR indicates a suppressive role of EGFR.

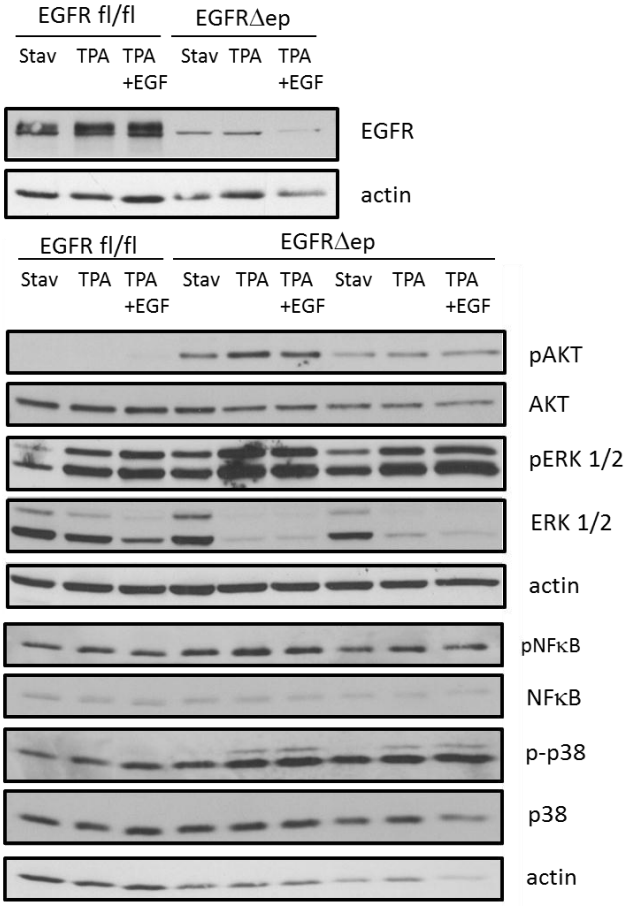


Figure 15 I Western blot analysis of keratinocytes stimulated in vitro with TPA or TPA and EGF. Analysis of phosphorylation of AKT, Erk1/2, NFκB and p38 in protein lysates from keratinocytes isolated from EGFR$^{fl/fl}$ and EGFR$^{\Delta ep}$ mice. Cultured keratinocytes were starved for 24 hours prior stimulation with TPA or TPA and EGF.

7. EGFR regulates infiltration of several immune cells after TPA treatment

Keratinocytes are responsible for skin homeostasis including the precise regulation of immune cell attraction to the leason by secretion of chemokines (Schafer and Werner, 2008). As mice defective in the EGFR pathway display skin inflammation, and due to the fact that it was shown that EGFR signaling affects the expression of different cytokines and chemokines *in vitro* (Pastore et al., 2005) EGFR signaling seems to play an important role in regulating immune responses of the skin.

As described above topical application of TPA on mouse skin is a well-known model for induction of cutaneous inflammation. To establish in which manner the lack of EGFR in the epidermis has an impact on the immune cell attraction, the skin of $EGFR^{\Delta epER}$ and $EGFR^{fl/fl}$ mice was treated with 10µg TPA/ 200µl acetone for 3 consecutive days. The O.C.T. embedded sections were double-stained with FITC and PE labeled antibodies detecting proteins like MHC class II and F4/80, GR1 and CD11b, CD11c and MHC class II, and CD3ε and langerin. Three independent batches were analyzed.

Generally, quantitative analysis of immune cell infiltration in TPA-untreated skin lacking the EGF receptor exhibited a lower number of macrophages and dendritic cells (Fig. 16A, C) compared to the control, contradicted to what was shown before (Fig.10A, C). One possible explanation could be that EGFR deletion in skin was insufficient, which could stretch the results. In wild type skin, TPA promotes a significant decrease of cells expressing MHC II (Fig. 16A), which could be a marker for macrophages, B-cells, LC and DC, CD11b, which are monocytes, and GR1/CD11b (Fig. 16B). In addition, a significant decrease of $CD3\varepsilon^+$ cells, which represent T-cells respectively, was detected (Fig. 16D). In $EGFR^{fl/fl}$ skin the attraction of several immune cells like macrophages, expressing MHC II and F4/80 (Fig. 16A), myeloid granulocytes, $GR1^+$ cells (Fig. 16B), DCs, expressing CD11c and MHC II (Fig. 16C), as well as Langerhans cells (Fig. 16D) seems to be TPA dependent.

In $EGFR^{\Delta epER}$ skin, TPA application resulted in a significant rise of macrophages ($F4/80^+$, $MHC\ II^+/\ F4/80^+$) (Fig.16A), monocytes ($CD11b^+$), early hematopoietic dendritic progenitor cells ($GR^+/CD11b^+$) (Fig.16B), DC ($CD11c^+$) (Fig. 16C), and a decrease of T-cells (Fig. 16D) compared to the untreated $EGFR^{\Delta epER}$ skin. Loss of EGFR in the skin does not affect TPA-mediated LCs infiltration (Fig. 16D).

These results show that EGFR regulates infiltration of several immune cells after TPA treatment which may indicates its regulatory role in the immune response during tumor promotion.

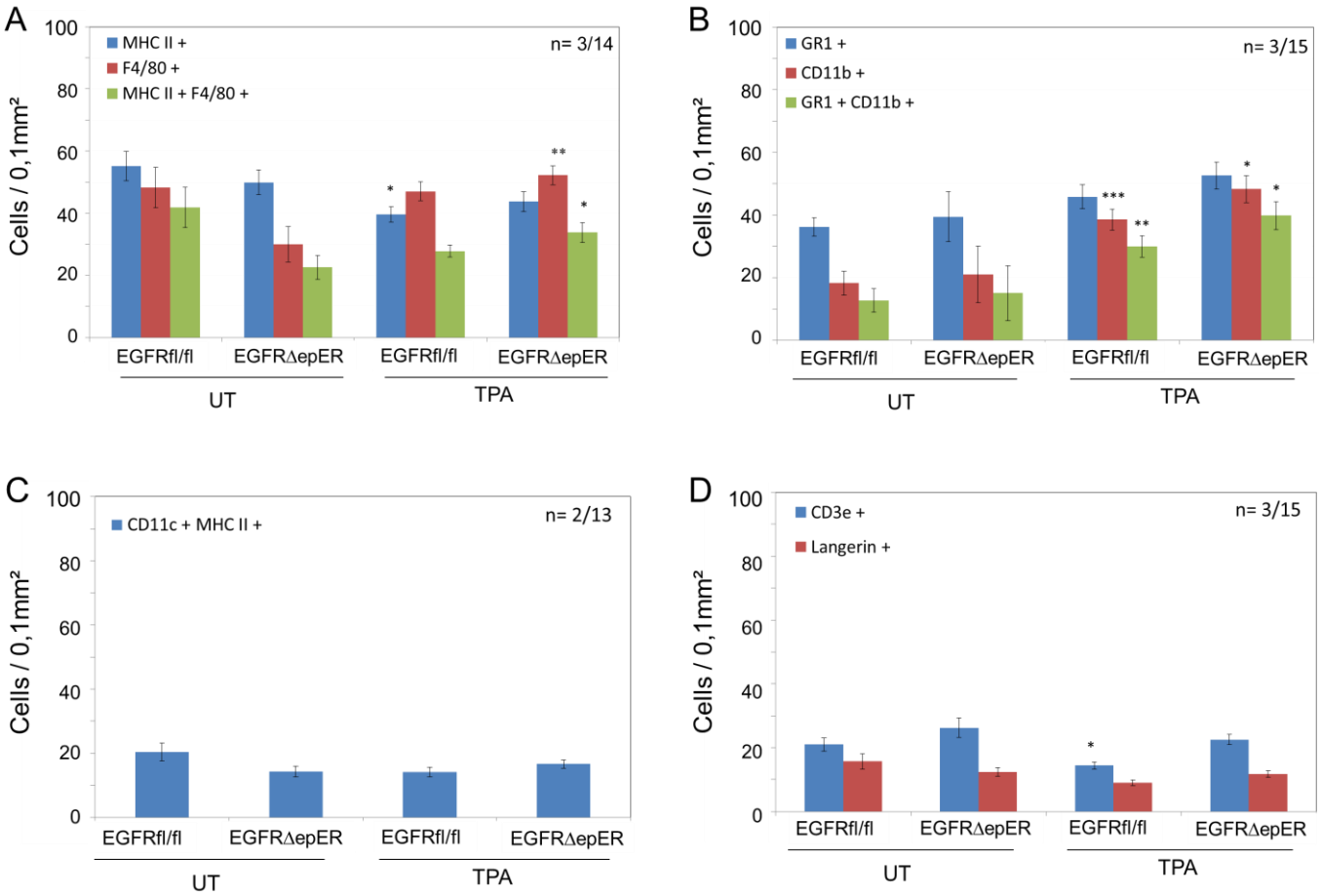


Figure 16 | Quantitative analysis of immune cell infiltration (cell/ 0,1mm^2) of untreated (UT) and TPA treated (TPA) back skin samples of EGFR$^{\Delta epER}$ and EGFR$^{fl/fl}$ mice (A-D). Quantification of MHC II$^+$ and/or F4/80$^+$ cells (**A**), GR1$^+$ and/or CD11b$^+$ cells (B), MHC II$^+$ and CD11c$^+$ cells (C), and of CD3ε$^+$ or langerin$^+$ cells (D) in EGFR$^{\Delta epER}$ and EGFR$^{fl/fl}$ skin. Data represent mean ± SEM. *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005

8. Inflammation in EGFR deficient mice is not sufficient to induce papillomas

Inflammation and carcinogenesis are tightly linked. The inflammatory microenvironment plays a very important role, because the formation and progression of tumors is dependent on the reactive stroma surrounding the cancer lesion (Moore et al., 1999). Mice lacking EGFR in epidermis display a general basic inflammation which might promote tumorigenesis. To investigate if the inflammation

found in EGFR mutant skin is sufficient to induce cancer, metabolic activation of procarcinogens was induced by a single topical application of 10µg DMBA, a common carcinogen, in seven week old mice ($EGFR^{fl/fl}$, $EGFR^{\Delta epER}$). Tumor development was monitored for 30 weeks leading to the conclusion that the inflammation resulting from loss of EGFR in the epidermis is not sufficient to induce papillomas after initiation (data not shown).

9. Induced inflammation in $EGFR^{\Delta epER}$ mice results in an earlier tumor occurrence

However, if DMBA treatment was followed by continuous application of TPA according to 2-stage skin carcinogenesis also $EGFR^{\Delta epER}$ mice developed tumors. For investigating the tumor incidence (= percentage of animals with tumors) and the tumor multiplicity (=numbers of tumors/mouse), seven weeks old $EGFR^{fl/fl}$ and $EGFR^{\Delta epER}$ mice were treated with a single subcarcinogenic dose of 10µg DMBA. After one week without treatment, tumor promotion was continued by TPA application twice a week for a time span of 30 weeks (Fig. 17).Interestingly, in contrast to what was published before (Casanova et al., 2002; Sibilia et al., 2000) mice lacking EGFR in the epidermis developed tumors earlier than wt controls.

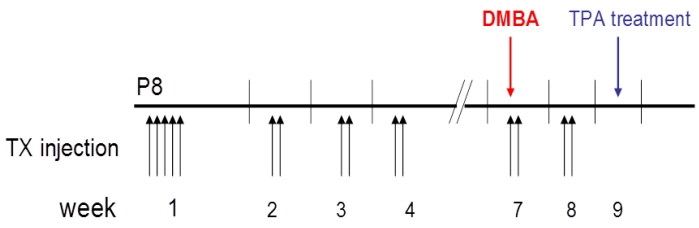


Figure 17 | Schematic illustration of two-stage carcinogenesis in $EGFR^{\Delta epER}$ mice. Tumor induction via DMBA at week 7 and promotion by TPA twice a week from week 9 on was accomplished. Tamoxifen was applied twice a week.

Mice lacking EGFR in the epidermis revealed an earlier tumor occurrence and the first tumor was detected on $EGFR^{\Delta epER}$ back skin eight weeks after the beginning of tumor promotion by TPA application. Moreover, all $EGFR^{\Delta epER}$ mice had developed tumors after 12 weeks; whereas 90% of $EGFR^{fl/fl}$ mice were tumor free at this time point and three weeks later 70% of $EGFR^{fl/fl}$ mice still had not developed any papillomas. Only after 17 weeks of promotion with TPA all remaining control mice evolved tumors at their back skin (Fig. 18A). These data suggest that in a 2-stage

skin carcinogenesis model genetic deletion of EGFR in keratinocytes could promote tumor formation. Further analysis of total and average tumor size reveal that EGFR$^{\Delta epER}$ mice papillomas had an initial growth advantage towards those evolving in EGFR$^{fl/fl}$ mice. However, at late time-points (week 20) tumors from mice lacking EGFR in the epidermis stopped growing and entered tumor stasis, whereas papillomas from wild-type mice constantly increased in size.

In summary, it can be stated that in EGFR$^{\Delta epER}$ mice early tumor formation might result from the skin inflammation, triggered by the absence of EGFR in epidermal cells (Fig. 18A). However, lack of EGFR negatively affects tumors volumes compared to tumors in wild-type controls (18 B, C).

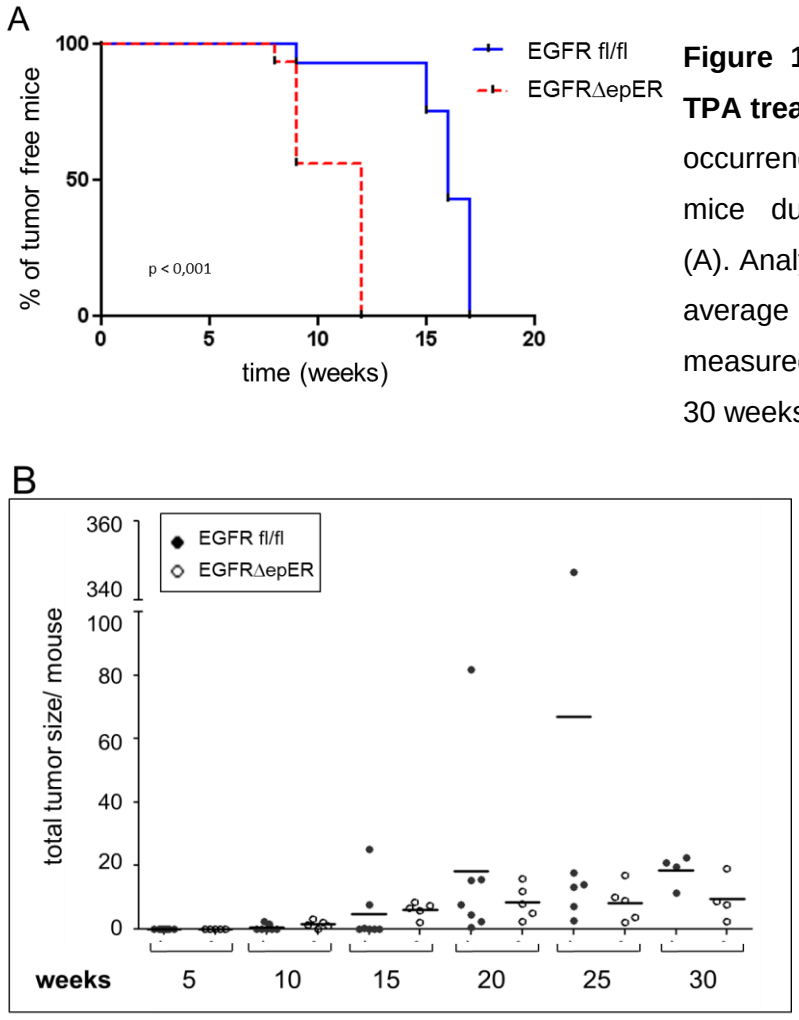
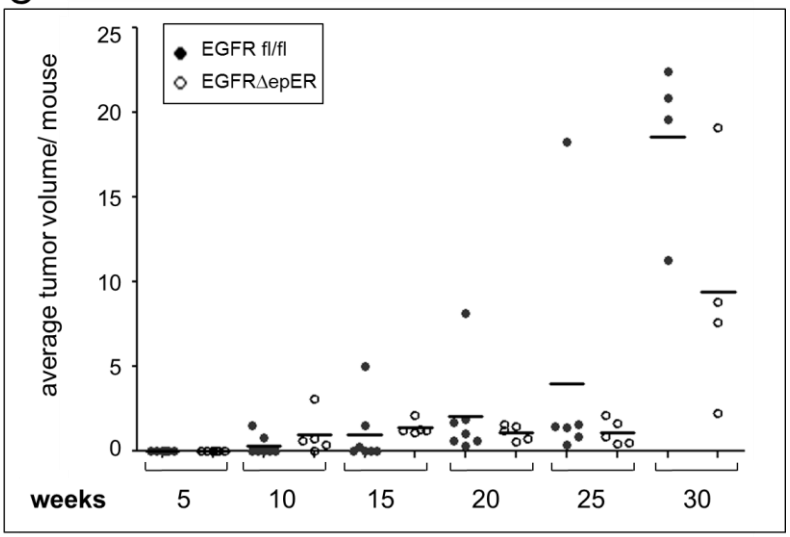


Figure 18 I Tumor development in TPA treated mice. Monitoring of tumor occurrence in EGFR$^{fl/fl}$ and EGFR$^{\Delta epER}$ mice during 2-stage carcinogenesis (A). Analysis of total tumor size (B) and average tumor volume (C) per mouse measured every five week for a total of 30 weeks (n= 5-7).

Data represent mean ± SEM. *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.0005

C



10. Tumors lacking EGFR display a higher proliferation

EGFR is overexpressed in many of tumors (Gullick, 1991; Habib et al., 2003) and EGFR signaling significantly affects survival of cancer cells (Lichtenberger et al., 2010; Sibilia et al., 2000). Moreover, dn EGFR tumors display a higher apoptosis rate, but no significant change in their proliferation rate (Casanova et al., 2002). Tumors from both $EGFR^{fl/fl}$ and $EGFR^{\Delta epER}$ back skin were isolated after 25 weeks of TPA treatment to investigate EGFR-dependent tumor survival in the skin. Furthermore, double staining for apoptotic and proliferating tumor cells were performed to investigate if TPA application has an impact on central and marginal tumorigenic tissue of $EGFR^{\Delta epER}$ and $EGFR^{fl/fl}$ mice.

Histological analysis of $EGFR^{fl/fl}$ suggests that tumors reveal a higher proliferation rate in the tumor center (Fig. 20A, B), and that in the marginal tumor tissue primarily apoptotic cells are detected (Fig. 21A/B). Interestingly, tumors lacking EGFR display strong proliferation, but less apoptosis in their central region (Fig. 20C) compared to wt controls after TPA treatment. Furthermore, $EGFR^{\Delta epER}$ marginal tumor tissue reveals no obvious difference in the apoptosis rate compared to $EGFR^{fl/fl}$. Generally marginal tumor tissue derived from $EGFR^{\Delta epER}$ shows a low number of proliferating and a high number of apoptotic cells (Fig. 21C).

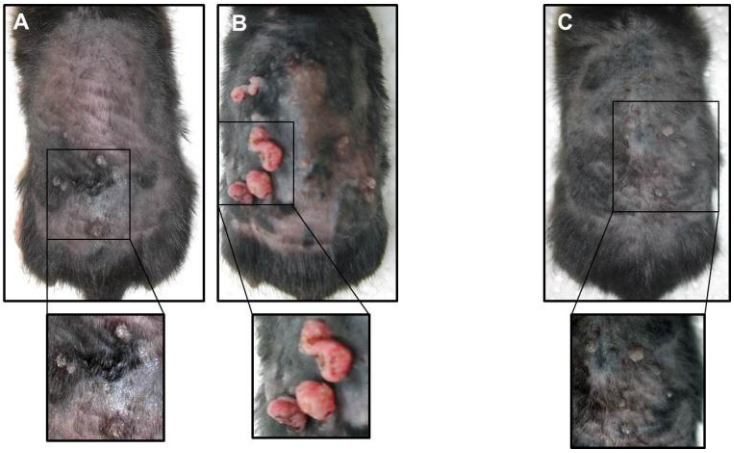


Figure 19 | Macroscopic appearance of back skin treated with TPA.
$EGFR^{fl/fl}$ (A, B) and $EGFR^{\Delta epER}$ (C) back skin treated with the tumor promoting agent TPA for 25 weeks. Tumor volumes in $EGFR^{fl/fl}$ (B) mice are bigger than in $EGFR^{\Delta epER}$ mice.

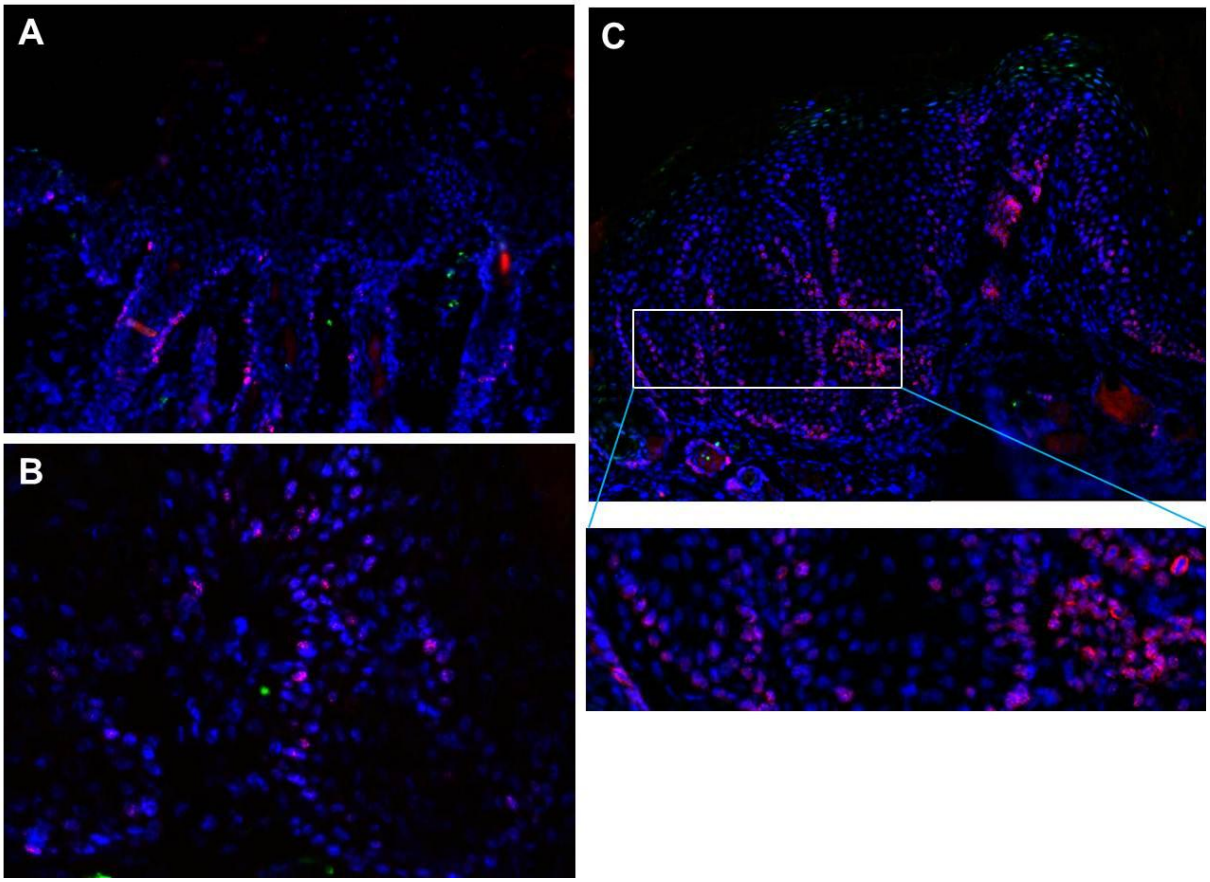


Figure 20 | Immunohistological analysis of central tumorigenic tissue in $EGFR^{fl(fl}$ and $EGFR^{\Delta epER}$ mice (A-C). Double staining with Ki67 antibody and ApopTag Detection Kit of tumor sections originating from $EGFR^{fl/fl}$ (A, B) and $EGFR^{\Delta epER}$ mice (C).

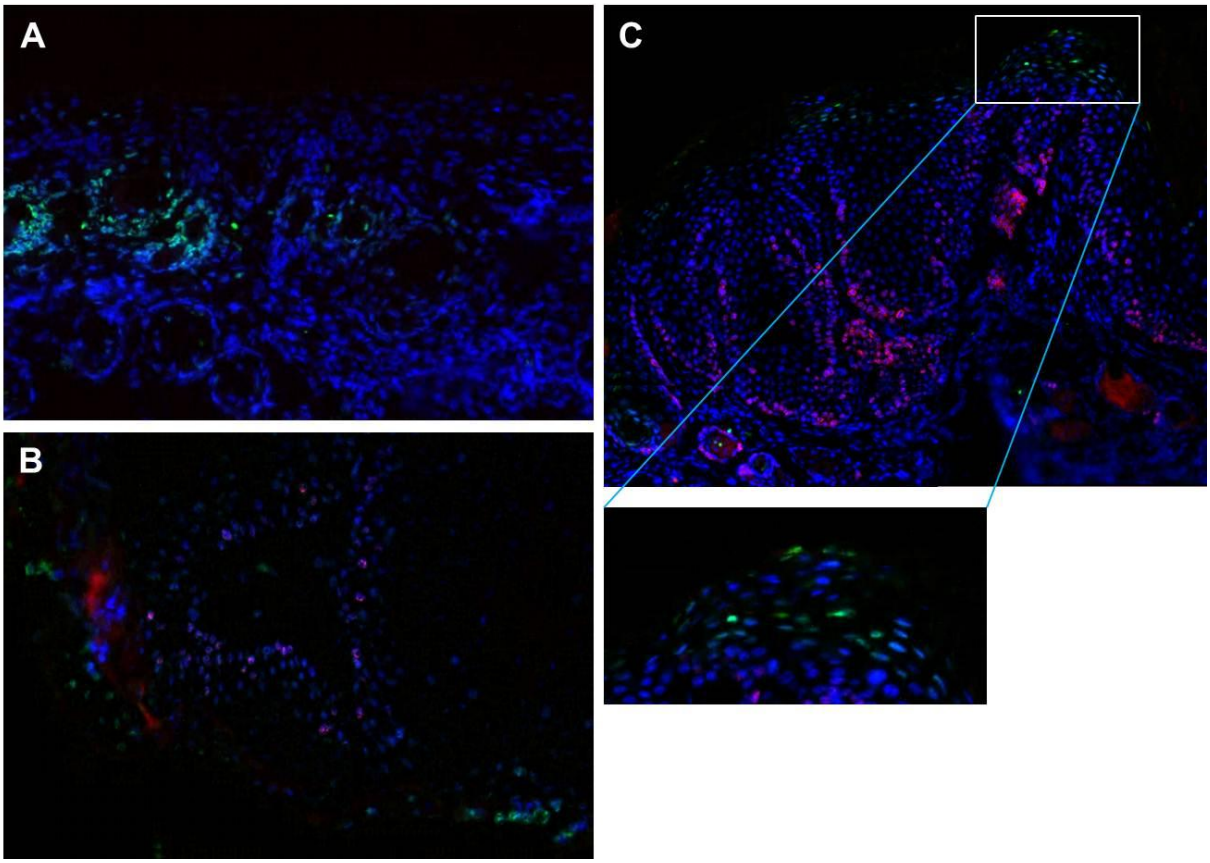


Figure 21 | Immunohistological analysis of marginal tumorigenic tissue in EGFR$^{fl(fl}$ and EGFR$^{\Delta epER}$ mice (A-C). Double staining with Ki67 antibody and ApopTag Detection Kit of tumor sections originating from EGFR$^{fl/fl}$ (A, B) and EGFR$^{\Delta epER}$ mice (C).

11. Regulation of immune cell composition by EGFR in tumor tissue

The process of wound healing, chronic inflammation and tumor development are very closely linked, not only by molecular but also by cellular parallels (Schafer and Werner, 2008). To gain insights into EGFR-dependent immune regulation in cancer, two EGFR$^{fl/fl}$ mice and one EGFR$^{\Delta epER}$ mouse were analyzed, which were treated with TPA for 25 weeks after DMBA induction. Figure 22 exhibit the immune cells composition present in the different regions (central, marginal) of a tumor. Skin samples were divided in tumor free tissue, which represents parts of the skin nearby the tumor, tumor center and tumor edge. Sections were double-stained with FITC and PE labeled antibodies detecting surface proteins like MHC class II and F4/80 (Fig. 22A-C), CD3ε and langerin, (Fig. 22D-F), GR1 and CD11b (Fig. 22G-I), and CD11c and MHC class II (Fig. 22J,K). Due to too less samples CD11c and MHC class II analysis of the tumor edge was impossible.

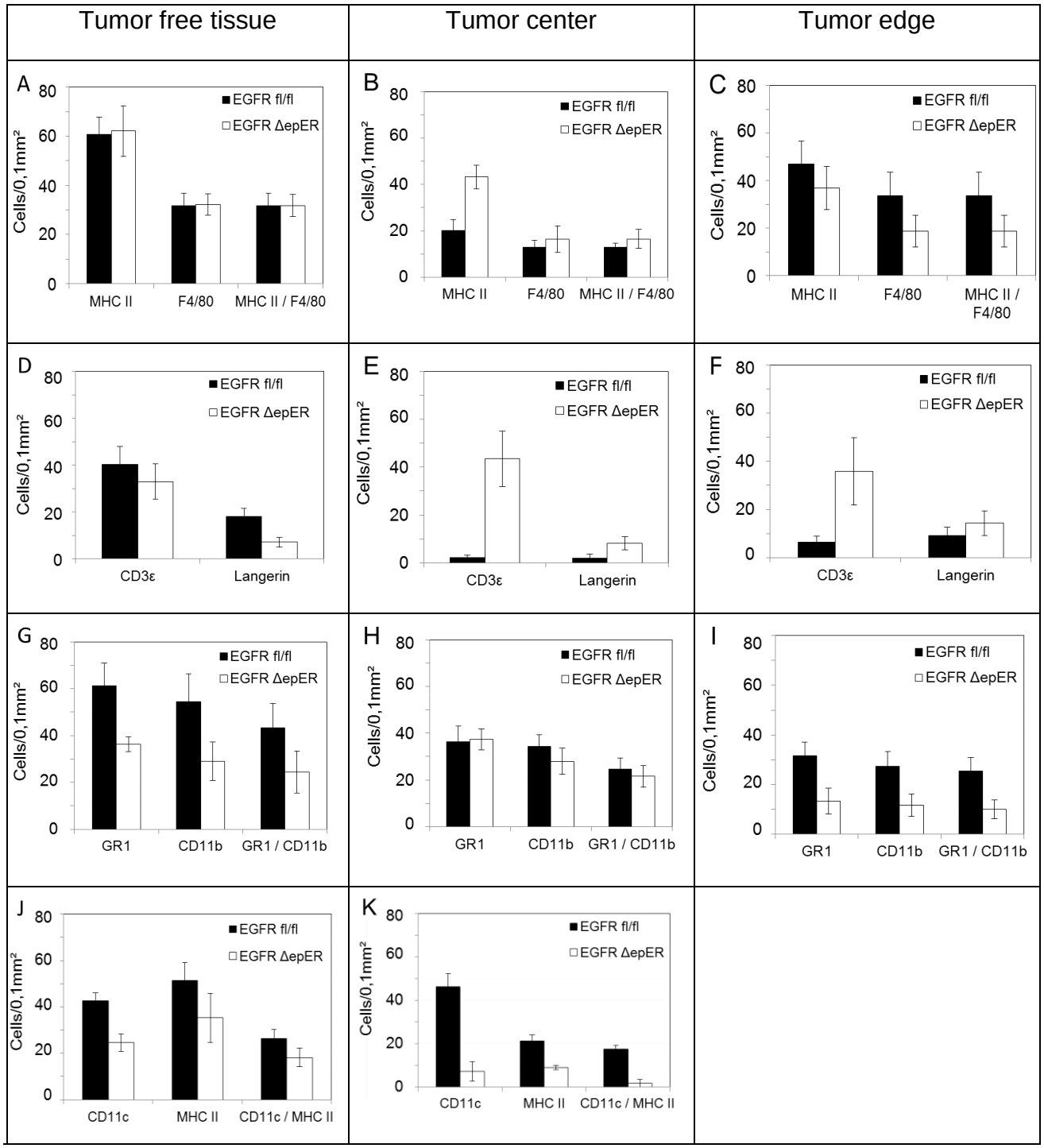


Figure 22 | Immunohistological quantification of immune cells in tumor free tissue, central and marginal tumor tissue (A-K). Isolated tumors from $EGFR^{fl/fl}$ and $EGFR^{\Delta epER}$ mice were stained for MHC II^+ and/or $F4/80^+$ cells (A-C), $CD3\varepsilon^+$ or $langerin^+$ cells (D-F), $GR1^+$ and/or $CD11b^+$ cells (G-I) and for MHC II^+ and/or $CD11c^+$ cells (J, K). Immune cells from tumor free tissue (A, D, G, J), central tumor tissue (B, E, H, K) and marginal tumor tissue (C, F, I) were quantified (n= 1-2). Data represent mean ± SEM.

Generally, quantitative analysis of MHCII$^+$ cells and macrophages (MHCII$^+$/ F4/80$^+$) in tumor tissue might be genotype-dependent (Fig. 22A-C). More precisely, it was shown that in EGFR$^{fl/fl}$ tumors MHC II$^+$ cells and the macrophages population of central differs from marginal tumor tissue. The amount of immune cells remains low in the tumor center (Fig. 22B) and is increased at the edge (Fig. 22B). It might be that the immune cells were attracted to the edge of EGFR$^{fl/fl}$ derived tumor. In EGFR$^{\Delta epER}$ papillomas MHC II$^+$ cells and macrophages were equally spread all over the tumor tissue.

The presence of T-cells (CD3ε$^+$) and LC (langerin$^+$) in tumor and tumor free tissue was also quantified (Fig. 22D-F). In wt mice only very few T-cells and LC were located within the tumors while many T-cells and LCs were located in tumor free tissue. Interestingly, while there were less LCs and T-cells in the tumor-free tissue of EGFR mutants mice compared to littermate controls, their numbers were significantly higher within the tumors (Fig. 22E/F).

Quantification of granulocytes (GR1$^+$), monocytes (CD11b$^+$) and early hematopoietic dendritic progenitor cells (GR1$^+$/ CD11b$^+$) (Fig. 22G-I) revealed that EGFR$^{\Delta epER}$ tumor free tissue displayed two-fold less cells than wt specimen (Fig. 22G). However, in wt tumors granulocytes, monocytes and early hematopoietic dendritic progenitor cells were less present than in tumor free tissue and equally spread all over the tumor tissue. In comparison, in papillomas from mutant mice the immune cells were concentrated at the tumor center (Fig. 22H), and no difference in immune cell abundance was detectable compared to the center of wt tumor tissue. Moreover, in tumor tissue lacking EGFR the amount of these immune cells was decreased at the tumor edge compared to the control (Fig. 22I).

As mentioned before due to too less samples, the analysis of DC (MHCII$^+$/ CD11c$^+$) infiltration in the tumor edge was impossible. Nevertheless, I found that DC members were dramatically decreased in EGFR$^{\Delta epER}$ derived central tumor tissue compared to the control (Fig. 22K).

In conclusion, the composition of the immune cell infiltrate is different in tumor tissue as well as the surrounding skin of wild-type and EGFR$^{\Delta epER}$ mice.

Discussion

The EGFR, one out of 4 receptors of the EGFR family, appears to be one of the most important and fascinating growth receptors. Its activation by multiple extracellular binding factors is responsible for many complex downstream pathways regulating a variety of cellular functions including proliferation, survival, migration and differentiation. Although the function of EGFR is still a widely uninvestigated field in science the receptor gains in importance in clinical applications and is therefore in the focus of many research groups. The proper expression of EGFR on the cell surface as well as its proper signal transduction in different organs and cell types is determining prenatal and postnatal development. EGFR affects premature differentiation of keratinocytes and influences osteoblast and chondrocytes differentiation negatively (Sibilia et al., 2007).

Many studies in mice lacking EGFR have confirmed that the EGFR signaling network is essential for skin development and its appendages (Sibilia et al., 2003; Sibilia and Wagner, 1995). Inhibitors of EGFR and ErbB2 cause suppression of hair growth in wt mice, and hair follicles remain in anagen (Mak and Chan, 2003). Mice with an epidermis-specific deletion of EGFR fail entering anagen-catagen-transition, the length of hair follicles is highly reduced, and, thus reveals a delay in HF cycling. Furthermore, I was able to show that in EGFR mutants proliferation of transit-amplifying cells is significantly decreased in hair follicles during anagen compared to their littermate controls. Interestingly, loss of EGFR causes a higher and constant level of apoptosis in HF of EGFR deficient skin. Evidence that EGFR mutant mice loose most hair follicles over time came from the analysis of $hEGFR^{KI/KI}$ mice, which display a progressive HF degeneration which might be by the massive inflammatory infiltrate in the skin, consisting mainly of macrophages, lymphocytes, neutrophils and multinucleated giant cells (Sibilia et al., 2003). Histological analysis of skin biopsies from $EGFR^{\Delta ep}$ mice exhibited a strong inflammation of both epidermis and dermis. Preliminary data have revealed that macrophages, myeloid granulocytes, monocytes and dendritic cells are significantly increased throughout the different stages of HF morphogenesis and HF cycle. Although previous unpublished results of the lab had shown that in the epidermis of EGFR deficient mice there were a large number of Langerhans cells and αβT-cells. However, quantification at different time points of hair follicle morphogenesis elucidated no significant difference in the dermis or

epidermis. Nevertheless, CD3ε^{+} T-cells are significantly increased in EGFR$^{\Delta ep}$ skin biopsies at postnatal day 19 only, while Langerhans cells infiltration is exclusively increased at postnatal day 10. The results obtained in this study confirmed the strong influence of EGFR balancing hair follicle morphogenesis and cycle, and immune responses of the skin.

EGFR plays a critical role in inflammation and epithelial tumorigenesis, two closely linked cellular processes. Previous studies confirmed mesenchyme-epithelium alterations and increased inflammation by abrogation of EGFR function in the skin (Luetteke et al., 1993; Murillas et al., 1995) and EGFR overexpression in epidermal tumors (Casanova et al., 2002; Hunts et al., 1985). Investigation of the functional effect of EGFR signaling on keratinocyte proliferation and survival revealed that EGFR deficiency reduces TPA-dependent hyperplasia as well as epidermal proliferation and apoptosis. Consistent with the decreased level of apoptosis in EGFR deficient skin, *in vitro* analysis of transgenic keratinocytes examine, that EGFR depletion enhances TPA-mediated AKT activity, although further analysis of mice genetically modified for EGFR expression indicate that EGFR acts as survival factor in epidermal tumors by initiation of the anti-apoptotic AKT pathway (Sibilia et al., 2007). Surprisingly, TPA-mediated ERK1/2 activation is EGFR dependent, despite the decreased proliferation rate. NFκB and p38 protein expression seemed to be EGFR independent. Due to the fact that EGFR signaling influences the expression of different cytokines and chemokines *in vitro* (Pastore et al., 2005), EGFR potentially plays an important role in the regulation of the immune response during tumor promotion. There is increasing evidence that the EGFR pathway has an important impact on the inflammatory reactions in the skin in presence of a tumor promoting agent. The results in this study demonstrate that EGFR deficiency increases TPA-dependent attraction of monocytes, early hematopoietic dendritic progenitor cells, T-cells and dendritic cells. Furthermore, TPA-induced Langerhans cell and macrophage attraction is EGFR independent. However, the effect of EGFR signaling on TPA-dependent expression of several chemokines and cytokines by keratinocytes and its influence on the inflammatory response needs to be further examined.

Moreover, I could show that the basic inflammation found in EGFR mutants is not sufficient to induce and/or promote papillomas. However, upon 2-stage skin carcinogenesis with DMBA/TPA, tumor formation does occur in EGFR mutant mice. Interestingly, in contrast to what was published before (Casanova et al., 2002; Sibilia

et al., 2000) EGFR mutant mice develop tumors earlier than wt controls. More precisely, it could be shown that tumor formation might result from skin inflammation triggered by the absence of EGFR in epidermal cells. Importantly, the analysis of total and average tumor size reveals growth advantage of papillomas from $EGFR^{\Delta epER}$ mice towards those evolving in $EGFR^{fl/fl}$ mice in the early stages of tumor formation. However, tumors from EGFR mutant mice enter tumor stasis and remain rather small, whereas papillomas from wt mice constantly increase in size. Additional studies are required to determine the influence of EGFR on tumor development.

Analysis of wt tumors and those derived from EGFR deficient mice revealed that in the central tumor tissue of EGFR mutant mice the proliferation rate is increased while apoptosis is decreased in comparison to wt controls. In marginal tumor tissue no obvious difference in the apoptosis rate was perceived. A very interesting finding is that the analysis of central and marginal tumor tissue revealed a different subset of immune cells in wt and EGFR mutant mice: the number of dendritic cells and macrophages is decreased in tumor tissue and they are spread all over it compared to controls. The number of both T-cells and Langerhans cells in papillomas lacking EGFR is much higher than in papillomas of wt, while the abundance of granulocytes, monocytes and early hematopoietic dendritic progenitor cells reveals no differences compared to controls. These results suggest that expression of EGFR in tumor tissue plays a critical role in the regulation of immune-cell infiltration.

Additional studies are required to determine how the complex EGFR signaling network regulates these responses to influence inflammation and tumor development, respectively. Deeper understandings of the impact of EGFR on skin inflammation will support the clinical progress of developing anti-EGFR therapies in skin cancer, as development of inflammatory skin eruptions and abnormal hair growth is often therapy-limiting.

Materials and Methods

Mouse strains

Conditional EGFR mice ($EGFR^{fl/fl}$) (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.
$EGFR^{-/-}$ (Sibilia and Wagner, 1995) and $EGFR^{wa2/+}$ and $EGFR^{wa2/wa2}$ were available in the laboratory. The Wa-2 EGFR allele contains a T-to-G transversion mutation in the sequences encoding the tyrosine kinase domain resulting in a substitution of a glycine for a highly conserved valine at position 743 in the third kinase subdomain. The ability of wa2 EGFR to phosphorylate an exogenous substrate is reduced by > 90% compared with that of the control receptor (Luetteke et al., 1994).
Mice were kept in the animal facility of the Medical University of Vienna in accordance with institutional policies and federal guidelines.

Tamoxifen treatment

To induce EGFR deletion K5-$CreER^{T}$ transgenic $EGFR^{fl/fl}$ mice were injected intraperitoneally with 1 mg 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. Deletion efficiencies were analysed by Western blot analysis.

Two-stage carcinogenesis and measurement of rising tumors

7-week-old $EGFR^{\Delta epER}$ mice and littermate controls were treated with 10µg DMBA. After one week intermission TPA treatment (5µg per mouse and day) was initiated and continued consequently twice a week. Tumors were measured with a digital caliper every second week.

Isolation and 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 and incubated for 1 hour at 37°C. The epidermis was separated from the dermis and placed into

DNAse-Medium (low calcium medium containing 8% FCS and 250µl/ml DNAseI and incubated for 20 min at 37°C shaking. The suspension was filtered through a 70µm cell strainer (Becton Dickinson) & cells were collected by centrifugation. Cells were washed once with DNaseI-medium & then seeded at a density of $6x10^6$ cells/100mm dish onto vitrogen-fibronectin coated dishes or were directly processed to protein lysates or RNA. Incubate cells at 32°C, 5% CO_2 and change medium every 2-3 days. For in vitro deletion of EGFR keratinocytes isolated from $EGFR^{\Delta epER}$ mice were cultured in medium containing 100nM tamoxifen. When cells reached 80% confluency cells were starved for 24hr, and stimulated with 100ng/ml TPA or 100ng/ml TPA/ 20ng/ml EGF for 5 minutes, they were kept on ice, washed properly with ice cold PBS and were processed to protein lysates or RNA.

Preparation of epidermal protein lysates and Wester Blot analysis

Epidermal cells kept on ice were washed with PBS and lysed with buffer containing 50mM Hepes pH 7,3, 150mM NaCl, 10% Glycerol, 1% Triton X-100, 1mM EDTA, 10mM $Na_2P_2O_7$, 10µm/ml Aprotinin, 10µm/ml Leupeptin, 25mM NaF, 1mM $NaVO_3$, 1mM PMSF, 20mM PNPP, H_2O; and centrifuged 10 min at full speed at 4°C. 40µg protein were separated by SDS-PAGE and transferred to PVPF membranes (Millipore). Western Blot was performed according to standard procedures (Sibilia et al., 2000) using the following antibodies: anti-EGFR (Cell signalling #2232; Santa Cruz Biotechnology); anti-phospho-AKT and anti-AKT (Cell signalling #4051); anti-phospho-NFκB and anti-NFκB; anti-phospho-p38 and anti-p38; anti-phospho-ERK1/2 and anti-ERK1/2 (NEB #9101L,); anti-actin and anti-tubulin (Sigma), and secondary antibodies from Jackson laboratories.

Isolation of genomic DNA

3-5 mm of mouse tail, small pieces of tissue or cell pellets were incubated overnight in 500µl Tail buffer (50 mM Tris-HCl pH8; 100mM EDTA; 100mM NaCl; 1% SDS and 0,5 mg/ml proteinase K) at 55°C. 200µl of saturated (6M) NaCl were added, the suspension was mixed and centrifuged 5 min at full speed. The supernatant was transferred to a new tube, 500µl isopropanol was added, everything was mixed 2 min on the shaker and again centrifuged for 5 min at full speed. After washing the pellet with 1ml 70% ethanol the DNA pellet was air dried. Finally, the DNA pellet was

resuspended in 300-400µl of TE (10mM Tris pH 7,6; 1mM EDTA) depending on the pellet size.

Restriction digest of genomic DNA

3µl genomic DNA were digested with Hind lll restriction enzyme (1,5µl Hind lll; 1,5µl 10x restriction buffer B; 9µl H_2O) and separated by electrophoresis on a 0,8% agarose gel.

Detection of the CRE transgene by PCR

1µl DNA solution and 24µl master mix, containing 2,5µl GB buffer; DMSO; dNTPs (10mM); primer K5Cre1 (1,2µM); primer K5Cre2 (1,2µM); 11,25µl H_2O; and 0,25µl Taq polymerase (5U/µl) was mixed for analysing the mouse genotype. The following primers were utilized to detect the Cre transgene:

K5Cre1: (24bp) 5' CAT ACC TGG AAA ATG CTT CTG TCC 3'

K5Cre2: (24bp) 5' CAT CGC TCG ACC AGT TTA GTT ACC 3'

Thermal cycle conditions:

94°C – 2 min

35x (94°C – 30 sec, 53°C – 45 sec, 65°C – 1 min)

65° - 5 min

4°C ∞

PCR products were separated on a 2,5% agarose gel. K5Cre positive mice show a 500bp band.

Total RNA isolation and RT analysis

Total RNA from epidermis, cultured epidermal cells, were isolated with TRIzol Reagent (Invitrogen). cDNA synthesis was performed 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 LightCycler 2.0 System (Roche).

Histological analysis

Skin biopsies were embedded in OCT and cryopreserved or fixed in 4% paraformaldehyde and embedded in paraffin and cut into 5µm section. Sections were

deparaffinized with xylene, rehydrated in an ethanol series (100%x2, 90%, 80%, 70%, 30%) and incubated in water as long as necessary. In case of cryosections, the slides were incubated in ice cold acetone at -20°C for 20min, air dried and washed with PBS.

For hematoxylin/ eosin staining the slides were incubated 5 min in freshly filtrated hematoxylin, washed 3 times in water, dipped into HCl water for 3-5 seconds and again washed 2 times. After an ethanol series (30%, 70%, 80%) the sections were incubated in eosin solution for max. 45 seconds. Then the samples were further dehydrated (90%, 100% ethanol and xylem) and mounted with Entellan.

For Ki67 staining the sections were incubated in an antigen retrieval solution (DAKO) in the microwave for 20 min and were washed 2 times with PBS. Next the slides were blocked with 10% goat serum/ PBS for 20 min and incubated with αKi67 antibody (1:1000, Novacastra) at 4°C over night. In the following steps the slides were washed with PBS, incubated with the secondary antibody anti-rabbit Alexa594 (1:5000; Jackson Laboratories) for 1 hour and washed again. Biopsies were counterstained with Hoechst (1:1000) for 20 min and mounted with Fluoprep and were stored in the dark.

For detection of various immune cells, sections were blocked with 10% goat serum/ PBS for at least 20 min at RT. Afterwards sections were washed with PBS and were incubated with antibodies from Biolegend diluted in 2% BSA/ PBS over night at 4°C:

MHC class II–PE (1:300) / F4/80-FITC (1:100)

GR1-PE (1:200) / CD11b-FITC (1:100)

CD11c-PE (1:200) / MHC class II-FITC (1:150)

CD3ε-PE (1:100) / Langerin-FITC (1:400)

In the following steps the biopsies were stained with Hoechst (1:1000) for 20 min and mounted with Fluoprep and were stored in the dark.

Images were obtained with a Nikon eclipse 80i microscope; histomorphometric analysis was performed with Lucia software.

ApopTag® Fluorescein In Situ Apoptosis Detection Kit and Ki67 staining

Cryo sections were air dried for 5 min, then fixed with 1% paraformaldehyde in PBS for 10 min and washed two times with PBS for 5 min. All this steps were done at RT. The slides were post fixed with ethanol-glacial acid (2:1) for 5 min at -20°C. They

were washed again twice with PBS for 5 min at RT. The following incubation steps were performed in a humid chamber. Sections were incubated with equilibration buffer for 5 min at RT and afterwards with TdT Enzyme solution (70% Reaction Buffer; 30% TdT Enzyme) for 1 hour at 37°C according to the manufacturer's suggestion (ApopTag® Fluorescein In Situ Apoptosis Detection Kit, Millipore). Sections were further incubated with the Stop/Wash Buffer for 10 min at RT and finally they were washed three times with PBS for 1 min.

Biopsies were blocked with 10% goat serum/PBS for 20 min at RT, incubated with a 1:100 dilution rat-αmouse-Ki67 (DAKO, Clon Tec 3) in 2% BSA/PBS at 4°C over night, washed with PBS, incubated with the secondary antibody goat-αrat-Alexa594 (Jackson Laboratories; 1:500 in 2% BSA/PBS) for 1 hour at RT and washed again.

Finally, sections were incubated with Anti-Digoxigenin Conjugate for 30 min at RT, washed four times with PBS and counterstained with DAPI or HOECHST and were mounted with Fluoprep.

Epidermal ear sheet preparation and staining

Ear sheets were separated, placed hairy side up onto 3,8% ammoniumthiocyanate/ PBS and incubated for 25 minutes at 37°C. Epidermis was separated form dermis, washed in PBS and fixed in acetone at RT for 15 minutes. Epidermal sheets were washed in PBS and stored in 2%BSA/PBS at -20°C. For epidermal sheet staining ear sheets were blocked with 10% goat serum/PBS for 20 minutes at RT and incubated with antibodies diluted in 2% BSA/PBS at 4°C over night (Langerin-FITC 1:400; MHC II-PE 1:300). The samples were counterstained with Hoechst for 20 minutes at RT and mounted hair side down with Fluoprep (Bio Merieux).

Statistical methods

Most of the 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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Curriculum Vitae

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Work experience

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August 2009 – August 2010	Scientific assistant in the group of Dr. M. Sibilia (Diploma Thesis)
August 2009	Tutor at the Vienna Open Lab (dialog gentechnik)
November 2008	Scientific assistant in the group of Dr. V. Jantsch-Plunger (Chromosome Biology)
May/ June 2008	Internship at Affiris AG: Identification and preclinical development of atherosclerosis vaccine
April 2008	Scientific assistant in the group of Dr. J. Seipelt (Infection Biology)
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November/ December 2006	Scientific assistant in the group of Dr. K. Kuchler (Infection Biology)
August 2005	Internship at Baxter: Classic & Photometric Analytics CPA

Mai 2003 – August 2006	WienIT EDV Dienstleistungsgesellschaft mbH & Co KG (kurz: WienIT) – Stabsstelle Recht

Education

1991 – 1995	Private elementary school PP. Piaristen
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2003 - 2011	University of Vienna: Study of Molecular Biology
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Skills

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