Tumor Necrosis Factors and Chemokines in Hair Development

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Tumor Necrosis Factors and Chemokines in Hair Development

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"The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' but 'That’s funny...’"

Isaac Azimov (1920-1992)
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Abbreviations

A20/Tnfaip3  Tumor necrosis factor alpha-induced protein 3
BCMA  B-cell maturation antigen
Bmp  Bone morphogenetic protein
BmpR  Bmp receptor
C57BL/6  Mouse strain
CK1  Casein kinase 1
Co-Smad  Common partner Smad
CTGF  Connective tissue growth factor a.k.a. Ccn2, Cyr61 and  Nov
CRD  Cysteine rich domain
Crinkled  Spontaneous Edaradd-null mouse strain
Dkk  Dickkopf
DD  Death domain
Dhh  Desert hedgehog
Downless  Spontaneous Edar-null mouse strain
Dsh  Dishevelled
E  Embryonic day (after fecondation)
Ectodin  Wnt modulator in surface ectoderm, a.k.a. Wise, Sostdc1
ED  Ectodermal dysplasia
Eda  Ectodysplasin, also transcript Eda-A1
Edar  EctodysplasinA1 receptor
Edaradd  Edar associated death domain
ERK  Extracellular signal-regulated kinase
Fadd  Fas associated death domain
FGF  Fibroblast growth factor protein family
FGFR  Fibroblast growth factor receptor
FVB  Mouse strain
Fzd  Frizzled
GITR  Glucocorticoid-induced tumor necrosis factor receptor
Gli  Glioma-associated oncogene homolog
Gsk3β  Glycogen synthase kinase 3 beta
HED  Hypohidrotic ectodermal dysplasia
Hh  Hedgehog protein family
HSPG  Heparan sulfate proteoglycan
Ihh  Indian hedgehog
IkB  Inhibitor of κB (kappa light polypeptide enhancer in B cells)
IKK  IkB Kinase complex
IRS  Inner root sheath
JNK  cJun N-terminal kinase
K14  Keratin-14 promoter
Lef-1  Lymphoid enhancer-binding factor 1
LRP  Low-density lipoprotein receptor-related protein
Ltβ  Lymphotoxin beta
<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>NEMO</td>
<td>NFκB essential modulator a.k.a. IKKγ</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NMRI</td>
<td>Mouse strain</td>
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<tr>
<td>OMIM</td>
<td>Online Mendelian Inheritance in Man</td>
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<tr>
<td>ORS</td>
<td>Outer root sheath</td>
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<td>PCP</td>
<td>Planar cell polarity</td>
</tr>
<tr>
<td>Pitx</td>
<td>Paired-like homeodomain transcription factor</td>
</tr>
<tr>
<td>Ptc</td>
<td>Patched</td>
</tr>
<tr>
<td>PthRP</td>
<td>Parathyroid hormone related protein</td>
</tr>
<tr>
<td>R-Smad</td>
<td>Receptor-regulated Smad</td>
</tr>
<tr>
<td>sFRP</td>
<td>Soluble frizzled-related protein</td>
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<tr>
<td>Shh</td>
<td>Sonic hedgehog</td>
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<tr>
<td>Sleek</td>
<td>Spontaneous Edar-null mouse strain</td>
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<tr>
<td>Smad</td>
<td>Mammalian homolog for Drosophila gene: mother against decapentaplegic</td>
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<tr>
<td>Smo</td>
<td>Smoothed</td>
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<td>Sostl</td>
<td>Sclerostin-like, a.k.a. ectodin</td>
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<tr>
<td>Sostdc1</td>
<td>Sclerostin domain containing 1, a.k.a. ectodin</td>
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<tr>
<td>Sox</td>
<td>SRY-box</td>
</tr>
<tr>
<td>Tabby</td>
<td>Spontaneous Ectodysplasin-null mouse strain</td>
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<tr>
<td>TCF</td>
<td>T-cell factor</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor-beta</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TnFR</td>
<td>TNF receptor</td>
</tr>
<tr>
<td>Tnfrsf</td>
<td>Tumor necrosis factor receptor superfamily</td>
</tr>
<tr>
<td>Tradd</td>
<td>TNF associated death domain</td>
</tr>
<tr>
<td>Traf</td>
<td>TNF associated factor</td>
</tr>
<tr>
<td>Troy/Tnfrsf19</td>
<td>Tumor necrosis factor receptor superfamily, member 19</td>
</tr>
<tr>
<td>Xedar</td>
<td>Ectodysplasin A2 receptor</td>
</tr>
<tr>
<td>XL-HED</td>
<td>X-linked hypohidrotic ectodermal dysplasia</td>
</tr>
<tr>
<td>Wnt</td>
<td>Wnt protein family</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
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The names of genes and mRNAs are in *Italics*, whereas protein products are in regular type.
Summary

Several embryonic organs, such as the hair follicle, develop as appendages of the ectoderm, the outermost layer of the embryo. These organs develop as a result of reciprocal tissue interactions between the surface epithelium and the underlying mesenchyme. The first morphological sign of a developing hair follicle is a thickening of the epithelium called a placode. Several major signaling pathways are important for the development of hair and other ectodermal organs such as Wnts, fibroblast growth factors (Fgfs), Transforming growth factor-beta (TGFβ), Hedgehogs (Hh) and tumor necrosis factors (TNFs). This thesis focuses on the role of TNFs in hair development and more particularly on one member of the TNF superfamily: Ectodysplasin (Eda). Mutations in Eda pathway components including the TNF ligand Eda, its receptor (Edar), and downstream effectors essential for activation of transcription factor NFκB in mouse or human give rise to a disease called hypohidrotic ectodermal dysplasia (HED). HED is an inherited disorder characterized by impaired development of ectodermal organs such as hair, teeth and several exocrine glands. A hallmark of mouse HED (Eda null mouse) is the absence of primary hair placodes that form at embryonic day 14 (E14). In order to identify the direct target genes of Eda, we have performed a microarray analysis on genes differentially expressed upon short exposure to recombinant Eda protein on Eda null skin at E14. Several of the genes identified belong to the major signaling pathways mentioned above and interestingly, include also six chemokines that have not previously been associated with hair follicle morphogenesis.

The purpose of this study was to validate whether the upregulated genes were truly transcriptional target genes of Eda/NFκB and to study their functional relevance in ectodermal organogenesis, in particular in hair follicle development. Based on these studies, we were able to confirm some Wnt pathway members, such as Dkk4 and Lrp4, TNF family member A20, and two chemokines, cxc110 and cxc111, as likely direct target genes of Eda. It is shown that whereas Dkk4 and Lrp4 are expressed in all ectodermal organs, A20 and the two chemokines seem to be hair follicle specific. Further study of Dkk4 and Lrp4 during development led us to conclude that Wnt and Eda pathways interact closely to fine tune the development of hair and other ectodermal organs. The role of A20 seems to be restricted to the termination of NFκB signaling induced by the Eda pathway in hair follicles. The lack of cxc110 and cxc111 signaling during hair follicle formation leads to more widely spaced hair placodes. This work has revealed an important role of Eda during hair placode induction as a modulator of inhibitors and activators of the major pathways in order to direct the patterning of hair placodes.

In addition to Edar, an Edar-related TNFR named Troy is known to have an important role in hair placode formation; however, studies on some mouse models have suggested that additional TNFRs could be involved. Here I report also the earliest results concerning this enterprise.
Introduction

Hair development is a complex mechanism that still needs to be understood. Hair, teeth, mammary glands, and sweat glands are all skin appendages derived from the ectoderm, the outermost layer of the embryo. Hence, skin appendages are also called ectodermal organs and although their adult morphology differs greatly, their early development is quite similar both morphologically and molecularly (Mikkola, 2007). The initiation, development, and differentiation of all these organs depend on highly conserved pathways. This thesis aims at describing all the major pathways with a central role in the development of ectodermal organs, with focus on tumor necrosis factors (TNFs) and in particular one member of the TNF family: Ectodysplasin. Mutations in Ectodysplasin (Eda), its receptor, or its co-adapator gene in vertebrates give rise to malformations of many skin appendages including teeth, mammary and sweat glands, nails, and hair. In humans, malformations in three or more ectodermal organs characterize a disease called hypohidrotic ectodermal dysplasia (HED). Affected humans usually present sparse body and scalp hair, missing and/or malformed (conical) teeth, and reduced or absent sweating leading to hyperthermia, a life-threatening condition. The majority of human HED are inherited as X-linked recessive traits due to mutations in the EDA gene; hence, affected patients are mainly males.

In order to study the importance of Eda and other major signaling pathways in hair development, our laboratory is using the mouse as an animal model. Our best asset is the Eda-deficient mouse model Tabby, which has a phenotype that mirrors human HED syndrome as it shows one hair type missing, malformed hair shafts, misshapen and missing teeth, and malfunctioning, misshapen, or absent glands. Other spontaneous mouse strains Crinkled, Downless, and Sleek have a similar phenotype as Tabby and are caused by mutations in components of the Eda pathway. A characteristic of the Tabby phenotype is the complete absence of primary hair placodes, which translates in the adult into the complete absence of one type of hair. This thesis is based on experiments with Tabby skin, where Eda protein has been used to study the activation of Eda signaling and its downstream target genes in this process.

In this thesis, I give an overview of hair formation and cycling and the role of the major pathways in these processes. For clarity, I will concentrate on mouse development, as most of the research made in this area uses mouse as a model and conclusions concerning the interplay of the major pathways have primarily been made based on mutant mouse phenotypes.
REVIEW OF THE LITERATURE

1 Ectodermal organ development

1.1 Origin of the ectoderm

Fertilization of the egg is followed by a series of mitoses and cleavage of cells in order to form the morula in mammals. During gastrulation, cells migrate simultaneously and form the multilayered body plan of a given organism. Cells that will form the endoderm and the mesoderm migrate inside the embryo, whereas the ectodermal cells will form the outermost layer of the embryo and, during later stages of development, the neural tube, neural crest cells, and the surface ectoderm. The surface ectoderm layer will give rise to the epidermis, hair, nails, olfactory epithelium, mouth epithelium, eye lens, tooth enamel, and glands such as the mammary, sweat or meiboian glands. Therefore, these organs are called ectodermal organs.

1.2 Ectodermal organ development and morphogenesis

All ectodermal organs share common morphological features during the early steps of development. Initiation of ectodermal organogenesis requires the communication between the ectodermal epithelium and the underlying mesenchyme (Figure 1a). The first sign of ectodermal organ formation is a local thickening of the epithelium called a placode accompanied by the condensation of the underlying mesenchyme (Figure 1b). The placode will thereafter invaginate into (hair, tooth, and mammary gland) or evaginate outward from the mesenchyme (feather). The first signal initiating the development of the placode has yet to be discovered; however, it is thought that the mesenchyme sends this first signal in all ectodermal organs except during tooth development (Dhouailly, 1973; Kollar, 1970; Lumsden, 1988; Mina and Kollar, 1987)

To determine which tissue (epithelium or mesenchyme) is the source of the primary inductive signal in hair development, several tissue recombination studies have been performed. In short, tissue recombination involves the recombination of epithelium and mesenchyme isolated from different body regions of the mouse (hair-forming and non hair-forming regions) (Kollar 1970) or from different species such as chick and mouse (Dhouailly 1973). For example, Dhouailly’s interspecies tissue recombination studies showed that if the epithelium is collected from a region that will not form a hair follicle and recombined on chick mesenchyme from a feather-forming region, the epithelium will give rise to hair-like follicle structures. Similarly, hair-forming mouse mesenchyme (with pre-formed dermal condensate) combined with non feather-forming chick epithelium will induce feather-like bud formation. Hence, it seems that the mesenchyme gives the first cues to the epithelium to form an appendage and that the epithelium is responsible for the identity of the skin appendage it forms depending on the species (Dhouailly, 1973; Sengel and Mauger, 1976). In tooth, tissue recombination studies showed that the first signal inducing tooth formation is sent by the mandibular maxillar region (lower and upper jaw) epithelium and combining this mandibular epithelium onto
a non odontogenic mesenchyme led to tooth placode formation, whereas the reverse experiment did not (Mina and Kollar, 1987; Lumsden 1988).

This first inductive signal tells the epithelium to make an appendage. Afterwards, as the epithelium starts forming a placode it sends a message to the mesenchyme to condense. The subsequent development and morphogenesis of each organ is specific. I will focus on mouse ectodermal organ development in the following paragraphs. The mouse is a mammalian animal model whose embryonic development takes about 20 days after fertilization. Therefore, I will refer to the different stages of mouse development according to the days post-fertilization and the aspects of limb development for more accurate timing (Martin, 1990). For example, hair follicle development starts at embryonic day 14 after fertilization (E14).

1.2.1 Mammary gland morphogenesis

The mammary placodes start to develop along two parallel milk lines around mouse embryonic day 11.5 (E11.5). The milk lines are detectable between mouse limbs around E10.5 before breaking into five mammary placodes along the ventral lateral axis (Robinson et al., 2000; Sakakura et al., 1987). The most anterior mammary placode gives rise to mammary gland number 1 and so on towards the posterior part of the embryo. Each mammary placode pair appears sequentially during embryonic development: the first mammary placode to appear is number 3, followed by 4, then 1 and 5 simultaneously, and finally number 2 (Veltmaat et al., 2003; Veltmaat et al., 2004).

The mammary placode (Figure 1b) expands and invaginates into the underlying mesenchyme to form the mammary bud at E12.5 (Figure 1c). The bud is surrounded by the primary mammary mesenchyme, which is composed of three to five layers of mesenchymal cells (Chu et al., 2004; Veltmaat et al., 2004). The secondary mesenchymal tissue located posterior to the bud also starts differentiating. This structure consists of pre-adipocytes and forms the fat pad precursor cells (Figure 1d) (Sakakura et al., 1982; Veltmaat et al., 2003). In the female mice, the mammary bud remains relatively quiescent from E14 to E16, when ductal morphogenesis starts. At this stage, a cord of epithelial cells grows down and passes the primary mammary mesenchyme to enter the secondary mammary mesenchyme filled with pre-adipocytes (Sakakura et al., 1982). Consequently, the mammary epithelium starts branching to form the initial ductal tree (Figure 1e) (Sakakura et al., 1982; Veltmaat et al., 2003). This ductal tree continues to branch at a similar rate as the body increases in size until female puberty (Watson and Khaled, 2008). During puberty, the ductal tree continues to branch due to hormonal cues until it fills up the fat pad; concomitantly, the mammary epithelium gives rise to the typical nipple structure (Foley et al., 2001). During pregnancy the mammary gland completes its development by inducing the formation of alveoli to produce milk during lactation. After pregnancy, the alveoli undergo apoptosis and disappear, leaving the mammary gland to resemble its original post pubertal stage (Hennighausen and Robinson, 2001; Watson and Khaled, 2008).

In the male mouse, mammary gland development is arrested at E14. In fact, as the bud forms, the male hormones (androgens) will induce the condensation of the mesenchyme around the neck of the epithelial bud until the connection between the
Figure 1. Main steps in the development of ectodermal organs such as mammary gland and tooth. All ectodermal organs develop as a result of reciprocal tissue interactions between the epithelium and the mesenchyme (a). The epithelium invaginates and induces the condensation of the mesenchyme (b). Further invagination results in the formation of a bud, the aspect of which varies according to the organ (c). Further morphogenesis specific to each organ (d) gives rise to the adult organ (e).
epithelial stack and the underlying bud is severed (Dunbar et al., 1999; Veltmaat et al., 2003). The anlage will thereafter undergo apoptosis; consequently, male mice usually have no mammary gland or only very rudimentary mammary trees (Dunbar et al., 1999).

1.2.2 Tooth morphogenesis

The mouse dentition is composed of only two types of teeth, the continuously growing incisor and three molars separated by a toothless region called the diastema in each jaw quadrant. The mouse also has conical incisors, which are able to grow continuously because they do not form a root. However, the beginning of the development of both types of teeth is similar until cusp and root formation. Hence, for the purpose of clarity, I will describe only molar development (for more information on incisor development, see Tummers and Thesleff, 2003; 2009).

The first sign of tooth initiation is the formation of the dental lamina, a crescent-shaped thickening of the epithelium that runs along the upper and lower jaws in the E11 mouse embryo (Jernvall and Thesleff, 2000). One molar and one incisor placode appear at E12 in each quadrant of the upper and lower jaws within the dental lamina (Lumsden, 1988; Mina and Kollar, 1987). The placode will grow down into the underlying mesenchyme in order to form the tooth bud around E13 (Figure 1c). The tooth bud then forms a cap-like structure at the junction between the epithelium and mesenchyme called the enamel knot (Figure 1d) (Jernvall et al., 1994). The enamel knot is a signaling center consisting of tightly compacted cells that do not divide, but instead signal to neighboring cells (Jernvall et al., 1994). Hence, neighboring cells proliferate and the epithelium broadens bucco-lingually, resulting in a cap stage tooth anlagen formation at E14 (Jernvall et al., 1994; Vaahtokari et al., 1996). At this stage, the epithelium forms the lateral cervical loops and encases the underlying mesenchyme called the dental papilla. Continued growth and folding of the epithelium result in the acquisition of the shape of the tooth crown during the bell stage. The first enamel knot will undergo apoptosis (Vaahtokari et al., 1996) and secondary enamel knots will form during the bell stage to mark the future cusps of the tooth (Figure 1d) (Jernvall et al., 1994; Jernvall and Thesleff, 2000). The bell stage also witnesses the first cell differentiation of the tooth, as epithelial cells closest to the mesenchyme will give rise to enamel secreting cells called ameloblasts. Mesenchymal cells closer to the secondary enamel knot will differentiate into odontoblasts specialized in dentin secretion (Ruch et al., 1995). Cells situated more centrally will form the dental pulp, which can regenerate the dentin after injury (Figure 1e) (Magloire et al., 2001).

1.2.3 Hair morphogenesis

The hair shaft protruding from the human scalp or skin is composed of terminally differentiated keratinocytes (trichocytes), which are tightly compacted into a fiber. What is hidden under our skin, the hair follicle, is far more interesting. It is a sophisticated mini-organ not only responsible for hair formation, but its continuous cycling in a highly regulated and autonomous manner also generates new hair throughout life (Schneider et al., 2009). This means that the hair follicle is able to regenerate itself. Furthermore, it is
Review of the Literature

Involved in skin regeneration during wound healing (Ito et al., 2005; Levy et al., 2007). The coupling of hair follicle with the sebaceous gland also plays a role in dispersion of sebum to lubricate and waterproof the skin and hair.

Hair follicle induction mostly takes place during embryonic and perinatal development. Hair follicle development is divided into morphologically distinct stages (Figure 2) (Schmidt-Ullrich and Paus, 2005; Schneider et al., 2009). Here, I will focus on the key morphological appearances of the hair follicle during embryonic development and the final composition of hair follicles.

**Figure 2.** Main morphological stages in embryonic hair development. The first sign of hair development is the formation of the placode followed by the condensation of the underlying mesenchyme (a). The epithelium will further invaginate and form the germ, also called bud (b). During the peg stage, mesenchymal cells send a signal to induce differentiation of epithelial cells into hair matrix cells (c). During the bulbous peg stage (d), the bulb will develop along with the sebaceous gland and hair canal. Hair matrix cells differentiate into inner root sheath (IRS) cell populations and outer root sheath (ORS) cell populations.

Adapted from Schmidt-Ullrich and Paus, 2005
The first stage, placode formation, is characterized by the condensation of the basal cell layer of the epithelium. Hence, epithelial cells composing the placode become columnar and the mesenchymal cells condensate underneath the forming placode (Figure 2a) to form the future dermal papilla.

The second stage is characterized by the appearance of the hair germ or bud (Figure 2b). Epithelial cells proliferate and migrate deeper into the mesenchyme (Magerl et al., 2001) in order to form the hair peg (Figure 2c). The hair peg is characterized by differentiating epithelial cells. A stalk of mesenchymal cell gets trapped by the epithelium to form the dermal papilla. It is presumed that the dermal papilla sends the second mesenchymal signal to the surrounding epithelial cells in order to induce their differentiation. As a result, the cells surrounding the dermal papilla start dividing and form the hair matrix cells (Figure 2c) (Hardy, 1992; Millar, 2002).

In the bulbous peg stage (Figure 2d), the hair follicle bulb is formed. The postmitotic hair matrix daughter cells move upwards and differentiate either into hair cells (three cell types), inner root sheath (IRS) cells (three cell types), or a companion layer (Schmidt-Ullrich and Paus, 2005). A cross-section of the mature hair follicle reveals up to eight concentric cell layers, each one of them expressing a different set of keratins (Sperling, 1991). The three most central layers, the hair cuticle, cortex and medulla form the hair shaft. Formation of the hair canal is initiated in the bulbous peg stage and allows the hair shaft to reach the surface of the skin (Paus and Cotsarelis, 1999). Along with the hair canal, the sebaceous gland will develop and mature around birth.

The fully developed hair follicle is composed of two parts: the “permanent” part, closest to the epidermis, which does not cycle, and a lower part anchored deeper in the dermis that comprises about two third of the follicle and undergoes phases of apoptosis and regeneration, thus called the “cycling” part (Figure 3). Notably, the bulge marks the end of the permanent region of the hair follicle. The lowest part is characterized by a bulbar region, which surrounds the dermal papilla, the bulb (Figure 3) (Hardy, 1992; Millar, 2002).

### 1.3 Hair cycling

Once the first hair follicles are formed during embryonic development, the hair shafts grow and start forming mouse fur postnatally. The first hair follicle cycle is initiated at day 17 postpartum as the cycling region of the hair follicle starts regressing through cellular apoptosis (Figure 3) (Botchkarev and Paus, 2003). This stage is called the catagen phase and continues until the dermal papilla reaches the permanent region and the hair follicle is only composed of the hair bulge, dermal papilla, the old hair shaft (club hair), and sebaceous gland. When the regression is complete, a population of cells belonging to the lower portion of the bulge will differentiate to form the secondary hair germ progenitor cells above the dermal papilla (Figure 3) (Ito et al., 2004; Muller-Rover et al., 2001). The permanent region remains quiescent for several days during the first telogen phase. At the onset of a new anagen, the bulge cells which contain stem cells regenerate the whole lower part of the hair follicle (Cotsarelis et al., 1990). These cells divide and differentiate to give rise to every cell layer comprising the mature hair follicle. The anagen phase corresponds to a period of hair growth; hence, the hair bulb
Figure 3. Key stages of the hair cycle. The hair cycle is divided into three phases: anagen (growth phase), catagen (regression phase) and telogen (resting phase). Postnatal hair follicle morphogenesis leads to the elongation of the follicle into the skin and production of the hair shaft which protrudes from the skin. Once the hair is mature and has reached its maximum length, it enters the catagen phase. The cycling portion of the hair follicle regresses through apoptosis until the most distal part, the dermal papilla, reaches the bulge where the stem cells reside. When the regression is complete, a portion of bulge cells will differentiate to form the secondary hair germ progenitor cells above the dermal papilla. This minimal unit will stay quiescent until the hair growth is reactivated. The club hair is shed at the exogen phase which can happen at anytime.
keratinocytes proliferates in order to form the hair shaft (Figure 3). This phase can last from one to three weeks in mouse and determines the maximum length of the hair shaft. The point of maximum hair length is called Anagen VI (Muller-Rover et al., 2001). In the next cycle, a new hair shaft forms and emerges through the hair canal while the club hair remains in the same socket, thereby contributing to the density of the fur coat. The exogen phase which gives rise to the shedding of the club hair is not fixed, as the old club hair can be shed by mechanical forces or by an unknown molecular process at any time (Schneider et al., 2009).

After injuries, such as depilation, bulge cells will undergo apoptosis. As a consequence, secondary hair germ cells will differentiate and give rise to every cell layer comprising the hair follicle (Ito et al., 2004).

1.4 Mouse pelage hair types

The mouse fur is composed of four different hair types characterized by their shape, length and medullary composition (Figure 4) (Dry, 1926). These four types of hair are formed during three different waves of hair placode formation during embryonic development. The most prominent and least frequent hair type is guard hair, also called tylotrich or primary hair. It develops at E14 and represents between 2-10% of the mouse coat. Guard hair is straight and composed of two rows of medullary cells. Its length can vary from 0,8 to 1,2 mm and usually can be seen protruding from the mouse fur. Guard hair is a singular hair type because of the presence of two sebaceous glands per hair and a larger bulb. The second types of hair forming around E16 are both awl and auchene hairs. Awl hair is the thickest hair on the mouse coat. It is composed of 3-4 rows of medullary cells and is straight, whereas auchene hair has one bend. Awl and auchene vary from 0,5 to 0,8mm and account for 25-30% of the mouse fur. The most abundant hair type appears during the third wave of hair placode formation from E18 onwards. It is called zig zag hair.

Figure 4. Different mouse hair types. The mouse fur is composed of four types of hair. The guard hair is the longest and composed of two rows of medullary cells. Awl hair is shorter and consists of 3 to 4 rows of medullary cells. Auchene hair has one bend and is also composed of 3-4 rows of medullary cells. The most prominent type of hair is the zig zag, which has 3-4 bends and is composed of only one row of medullary cells.
hair and accounts for 60-70% of the whole fur. This hair type is responsible for the insulation of the mouse and usually is composed of 3 to 4 bends and only one row of medullary cells (Schlake, 2007).

2. Signaling pathways involved in ectodermal organ development

Many signaling pathways are involved in hair follicle and other skin appendage development. In this chapter, I aim to describe the most important ones. I will also briefly describe the outcome of modulating these pathways (loss or over-expression) during mouse ectodermal organ development except for hair development, which will be discussed in chapter 5.

2.1 Wnt signaling pathway

Wnt signaling is one of the most important pathways involved in embryonic development from gastrulation until organogenesis. In the adult it is responsible for maintenance of stem cell niches in many organs, and when deregulated may lead to cancer (Klaus and Birchmeier, 2008). The first Wnt family member was discovered in mammals because of its role in cancer development, as the mammary tumor virus was shown to induce the expression of Integration1 (Int1) and cause mammary gland tumors (Nusse and Varmus, 1982). In Drosophila, a wingless (Wg) mutation during embryonic development deprives flies from developing wings (Nusslein-Volhard and Wieschaus, 1980). As these two genes were found to be orthologous, the pathway was later termed the Wnt signaling pathway (Clevers, 2006).

Nineteen secreted Wnt proteins have been discovered in mouse based on the conserved cysteine residues and genome sequencing. Wnt proteins are characterized by a highly conserved cysteine that needs to be palmitoylated by the secreting cell to allow targeting at the cell membrane (Willert et al., 2003).

Wnt acts as a morphogen during development, which means that the protein is able to be secreted by the source cells and transported to form a long-range concentration gradient and act on distant cells in a concentration-dependent manner. The gradient triggers the expression of different target genes depending on different concentration thresholds sensed by the target cells (Wartlick et al., 2009). Because of Wnt protein insolubility, it has been postulated that this concentration gradient is mediated by active transport from the source cells, but the Wnt secretion mechanism remains unclear (Clevers, 2006).

The Wnt proteins signal through three different pathways: one canonical and two non-canonical pathways, also named planar cell polarity (PCP) pathway and Wnt/Ca2+ pathway mediated through receptor Ror2 or Ryk (Gordon and Nusse, 2006).

2.1.1 Canonical Wnt pathway

The canonical pathway is the most studied during development. It is based on the stabilization of β-catenin in the cytoplasm of the cell followed by β-catenin translocation
into the nucleus where it will induce the transcription of its target genes; therefore, this pathway is often referred to as the Wnt/β-catenin pathway (Figure 5).

When Wnt signaling is not active, β-catenin is associated with a degradation complex, which is composed of Axin, Adenomatous polyposis coli (APC), Glycogen synthase kinase 3 (GSK3) and Caseine kinase 1 (CK1). β-catenin is then phosphorylated by the kinases and sent to degradation by the proteasome (Figure 5a) (Clevers, 2006; Price, 2006). In the cell nucleus, Tcf/Lef family members (Tcf1,-3,-4 or Lef1) act as transcriptional repressors by forming a complex with Groucho proteins (Cavallo et al., 1998).

When Wnt signaling is activated, the secreted Wnt protein binds to its receptor Frizzled (Fzd), a seven-transmembrane receptor whose extracellular N-terminus contains a cysteine rich domain (CRD) responsible for Wnt binding efficiency (Lin et al., 1997). In order to transduce the signal, the Wnt/Fzd complex also needs a single pass signaling molecule: the lipoprotein receptor related protein 5 or 6 (Lrp5/6) (Pinson et al., 2000). Fzd can bind to Lrp5/6 at the cell surface and, upon Wnt binding, transduces the signal to Dishevelled (Dsh), GSK3β, Axin, APC and CK1α. When the Wnt canonical pathway is activated, Dsh is recruited to Fzd and phosphorylated (Yanagawa et al., 1995), while Axin binds to Lrp (Mao et al., 2001; Tolwinski et al., 2003) thereby destroying the β-catenin degradation complex. β-catenin stabilized in the cytoplasm translocates into the nucleus and binds to a Tcf/Lef family member, displaces Groucho from the Tcf/lef complex and promotes the transcription of its target genes (Behrens et al., 1996).

The R-spondin family members (Rspo 1-4) are secreted proteins that activate the Wnt canonical pathway by synergizing with Wnts to activate β-catenin. It has recently been found that Rspo can bind to Leucine-rich repeat containing G-protein receptors 4, 5 or 6 (Lgr4/5/6) in order to induce the canonical Wnt pathway through Lrp6

**Figure 5.** Wnt canonical pathway. When Wnt does not bind to its receptor, CK1 and GSK3β phosphorylate β-catenin, which is then targeted to the proteasome and destroyed. When Wnt binds to its receptor Frizzled (Fzd) and the co-receptor LRP, the complex activates the canonical pathway and Fzd phosphorylates Dishevelled (Dsh). LRP will recruit CK1, thus regulating the docking of Axin. The recruitment of Axin from the destruction complex will lead to the stabilization of β-catenin in the cytoplasm and its translocation into the nucleus where it will displace Groucho from the TCF/Lef1 complex and trigger the transcription of its target genes.
phosphorylation (Carmon et al., 2011; de Lau et al., 2011). Norrin is also an activator of Wnt pathway and binds specifically to Fzd4 in an Lrp5/6-dependent fashion (Xu et al., 2004).

Several potent families of Wnt pathway inhibitors have been characterized, including Wise family members such as Sclerostin (Sost), Sclerostin domain containing 1 (Sostdc1), and the Dickkopf family (Dkk1,-2,-4). Both families bind directly to Lrp5/6 (Itasaki et al., 2003). Dkks utilize their transmembrane receptor Kremen to form a complex with Lrp and induce the internalization of the complex into the cell rendering Wnt pathway inactive (Mao and Niehrs, 2003). However, the inhibitory activity of Dkks depend on kremens present on the cell surface, for example, Dkk4 requires Kremen2 to internalize Lrp5/6 in vertebrates (Mao and Niehrs 2003). Dkk4 has also been shown to be induced by canonical Wnt signaling during ectodermal organ development (Bazzi et al., 2007; Sick et al., 2006). Frizzled Related proteins (FRPs) that present the same CRD domain as Fzd (Hoang et al., 1996) can function as soluble extracellular inhibitors of Wnt ligands or can stabilize Wnt proteins to promote the activation of the Wnt pathway depending on the context (Logan and Nusse, 2004).

### 2.1.2 Non-canonical Wnt pathways

The non-canonical Wnt pathway does not seem to have a role in gene transcription, but rather in cell motility, embryonic axis formation, and cell polarity. Although this pathway still involves binding of Wnt on Fzd, the co-receptors involved are different as are the pathways they activate afterwards.

Non-canonical PCP/Wnt signaling also requires signal transduction through Dsh. In vertebrates, Wnt5a is considered to be a “classical” non-canonical Wnt ligand and can form a complex with membrane bound receptor tyrosine kinase: Ror2 and Vangl induce PCP and limb development in mouse. Wnt5a/Ror2 or Fzd2-4,6 induce the Wnt/Ca2+pathway, which is important for axonal path finding in the brain (De, 2011).

### 2.1.3 Role of Wnts in ectodermal organ development

During ectodermal organ development canonical Wnt signaling is active at multiple stages from placode induction to organ morphogenesis (Andl et al., 2002). Wnt gene expression can be found in ectodermal organs such as hair, mammary gland, and tooth.

Wnt10a and Wnt10b are found in the placodal epithelium of hair, tooth and mammary gland (DasGupta and Fuchs, 1999; Reddy et al., 2001; Sick et al., 2006; Veltmaat et al., 2004). Lef1 expression is also found in those developing placodes both in the epithelium and mesenchyme (Kratochwil et al., 1996; Zhou et al., 1995).

Study of the Lef1 null mutant mice was the first to show that Wnt pathway activation is important for ectodermal organ development, as tooth buds fail to further develop. Only the mammary gland placodes numbers 1, 4 and 5 are visible in the embryo but do not give rise to mammary glands in the Lef1 null adult (Boras-Granic et al., 2006; van Genderen et al., 1994).
Overexpression of the Wnt/β-catenin pathway can initiate tooth development on its own and induces enlarged and ectopic tooth placode formation (Jarvinen et al., 2006; Liu and Millar, 2010). Embryonic study of the mouse mutant showed abnormal tooth morphogenesis with extensive epithelial budding and supernumerary enamel knots (Jarvinen et al., 2006).

2.2 Transforming growth factor beta/Bone morphogenetic protein pathway

Transforming growth factor beta (TGFβ) superfamily members play important roles during embryonic development and morphogenesis by regulating cell growth, differentiation, and apoptosis (Yue and Mulder, 2001).

The TGFβ superfamily contains more than 40 structurally related members, e.g., Tgfβs, activins, Nodal, glial cell line neurotrophic factor (GDNF), and bone morphogenic proteins (Bmps), some of which are also referred as Growth and differentiation factors (GDFs) (Massague, 2000). Amino acid homology comparison between TGFβ proteins pinpointed highly conserved cysteine residues in the carboxyterminal region of those proteins (Pearce et al., 1999). The prototypic TGFβ isoform (Tgfβ1-3) and inhibitin β have nine cysteines, eight of which are involved in forming four internal disulfide bridges important for protein stabilization while the remaining cysteine forms interchain disulfide bridges between monomers (Hinck et al., 1996; Mittl et al., 1996; Schlunegger and Grutter, 1993). Bmps and GDFs usually have seven cysteines, of which six cysteines residues form three disulfide bridges responsible for protein stabilization. The active signaling molecule is typically formed through homodimerization requiring the seventh cysteine from each monomere to form a single interchain disulfide bond required for their activity (Chim et al., 2011; de Caestecker, 2004). All proteins form these dimers except for GDF3, GDF9, and GDF9B (Bmp15), which lack the seventh cysteine needed for dimerization (McPherron and Lee, 1993). Bmp proteins can form heterodimers (e.g. Bmp2/7, Bmp2/6), which seems to be a more potent activator of the Bmp pathway than the homodimer (Little and Mullins, 2009; Valera et al., 2010).

During embryonic development, the secretion of Bmp proteins is regulated by the diffusible binding proteins. Bmp proteins form a complex with Chordin (a Bmp inhibitor), which regulates the levels of expression of Bmp proteins by sequestration of the protein and promote the movement of Bmps in Xenopus embryos during the formation of the dorso-ventral axis, for example (Ben-Zvi et al., 2008).

2.2.1 Signal transduction

TGFβ superfamily ligands function in autocrine and paracrine manners and signal by binding to their cognate cell surface receptors (von Bubnoff and Cho, 2001).

TGFβ/Bmp canonical pathway

TGFβ receptors function as dimers that bind to two types of transmembrane serine/threonine receptors: type I (e.g BmpRI) or type II (e.g BmpRII). In order to activate TGFβ pathways, both types of receptors need to be expressed at the cell membrane, as TGFβ proteins bind only to an heterodimeric complex composed of type I and type II
receptors to form the active heterotetrameric receptor complex (Groppe et al., 2008). TGFβ signaling is mediated via Smad (Mammalian homolog of Drosophila mother against decapentaplegic) family members, which transduce the signal and induce the transcription of TGFβ target genes (Massague, 2000).

In mammals, the Smad family is composed of three groups: receptor specific Smads (R-Smad also called Smad1, -2, -3, -5, -8), common partner Smad (Co-Smad also called Smad4), and inhibitory Smad (I-Smad, Smad6 and -7). Smad1, Smad5 and Smad8 mediate signals from Bmps, whereas Smad2 and Smad3 transduce TGFβ/Activin/Nodal subfamily signals (Li et al., 2003; Massague and Wotton, 2000). The TGFβ/Activin/Nodal type I receptor phosphorylates and activates the Smad2/3 complex. Phosphorylated Smad2/3 will in turn form a complex with Smad4 and translocate into the nucleus to induce the transcription of TGFβ target genes (Heldin et al., 1997; Lagna et al., 1996).

Signaling through Bmp receptors leads to phosphorylation of the Smad1/5/8 complex, hence allowing complex formation with Smad4. Thereafter, the complex translocates into the nucleus and initiates the transcription of Bmp target genes (Heldin et al., 1997; Lagna et al., 1996).

Interestingly, Smad6 preferentially inhibits Bmp pathways, whereas Smad7 inhibits TGFβ/Activin/Nodal (Massague, 2000). Several secreted TGFβ family inhibitors have been identified. Follistatin is an inhibitor of Activin and Bmp pathways and does so by sequestrating the ligands thus rendering them unavailable for their receptors (Sugino et al., 1997). Noggin is an inhibitor of Bmp signaling along with Chordin and DAN proteins family members such as Gremlins, Dan, mouse cerberus-related protein (Cer1), and Dante (Pearce et al., 1999; Topol et al., 2000; Walsh et al., 2010). The inhibitor Tob seems to specifically antagonize Bmp2 signaling (Yoshida et al., 2000).

**Non-canonical Bmp pathway**

The non-canonical Bmp pathway does not involve Smad protein recruitment and seems to depend on BmpRs situated at the membrane. Studies on Bmp2 signaling have suggested that if the receptors are preforming a dimer, it will trigger signal transduction via the canonical Bmp-Smad pathway, whereas binding to the BmpRI with subsequent recruitment of BmpRII activates the Bmp-MAPK pathway, which is involved in the control of cell proliferation, migration, terminal differentiation, and cell death (Nohe et al., 2002; Siegel and Massague, 2003). Once BmpR complex is activated it will recruit the intracellular adaptor protein XIAP, which binds to TAK1 binding protein (TAB1) to finally activate TGFβ activated kinase 1 (TAK1) (Yamaguchi et al., 1999). TAK1 activates the Nemo-like kinase (NLK), which has been shown to inhibit phosphorylation of Tcf1/Lef1 transcription factors and downregulate Wnt/β-catenin-dependent transcription (Ishitani et al., 1999). TAK1 also activates p38, which is involved in Bmp-induced apoptosis (Shim et al., 2009; Zhang et al., 2000). Interestingly, Smads have been shown to bind TAK1 (Hoffmann et al., 2005), suggesting links between Bmp canonical and non-canonical pathways.
2.2.2 Role of Bmp signaling in ectodermal organ development

Loss-of-function mutations in Bmp2, Bmp4, BmpR1a, Smad1, Smad4, and Smad5 lead to early embryonic lethality due to multiple defects in developing mesodermal, ectodermal, and endodermal derivatives (Hogan, 1996; Itoh et al., 2000; Whitman, 1998). During placode formation, Bmp2 is expressed in the epithelium, whereas Bmp4 and Noggin expression is detected in the mesenchymal condensate (Hens et al., 2007; Lyons et al., 1990; Neubuser et al., 1997; Phippard et al., 1996; Vainio et al., 1993). In vitro studies have demonstrated that Bmp4 mediates the epithelial-mesenchymal interaction during early tooth morphogenesis (Neubuser et al., 1997; Vainio et al., 1993). A study of a mouse mutant with an epidermal specific deletion of BmpR1a showed a delay in tooth development after bud stage followed by regression of the tooth structures at E16 (Andl et al., 2004).

2.3 Hedgehog family

In mammals, the Hedgehog (Hh) family is composed of three proteins: Sonic Hedgehog (Shh), Indian Hedgehog (Ihh) and Desert Hedgehog (Dhh) (Echelard et al., 1993). The Hh name comes from the “spiked” aspect of the Drosophila larvae cuticle lacking this gene (Nusslein-Volhard and Wieschaus, 1980). The Hedgehog signaling molecules are secreted proteins that signal in an autocrine and a paracrine manner in order to control morphogenetic activities during ectodermal organ development (Chuong et al., 2000).

The processing of Hh protein has been studied in detail and seems to require several steps to process the protein into an active form. The active form of Shh is highly insoluble as one of the modifications forms a C-terminally cholesterol modified protein that binds to the plasma membrane (Porter et al., 1996). The final step of maturation requires the acetyl transferase Skinny Hedgehog (Ski in Drosophila, HHAT in humans) to ensure the palmitoylation of the active protein (Buglino and Resh, 2008; Chamoun et al., 2001; Lee and Treisman, 2001). Despite its insolubility, Shh acts as a morphogen during neurulation and limb development, for example (Hammerschmidt et al., 1997; Ingham, 1998; Murdoch and Copp, 2010).

2.3.1 Hh signaling pathway

In the absence of Hh ligand, Patched (Ptc), a 12-pass transmembrane protein and Smoothened (Smo), a seven-pass transmembrane member of the G-protein-coupled receptor (GPCR) superfamily form a complex preventing Smo from signaling to the cell. The mechanism by which Ptch represses Smo is currently unknown (Ingham et al., 2011; Taipale et al., 2002). In responding cells, Hh members bind to their receptor Ptc, (Stone et al., 1996). CDO (mammalian homolog of Ihog) proteins function as co-receptors with Ptc and are important for Hh signal transduction (Huangfu and Anderson, 2006; McLellan et al., 2008). The interaction of Hh and its receptor induces the release of Smo from the Ptc/Smo complex and triggers its activation. Smo activation modulates the repressor and activator forms of Gli zinc-finger transcription factors (Gli1-3 in mammals). In the “off” (Smo-inhibited) state of the pathway, Gli2/Gli3
are phosphorylated by protein kinase A (PKA), casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK3), targeting the proteins for proteasome-dependent processing (Nagase et al., 2007; Walterhouse et al., 1999).

All three Gli proteins have several signals for limited or complete proteolysis. Notably, degradation sequences (termed degrons) are found in Gli1 and Gli2 for binding to β-TrCP, an E3 adapter protein required for proteasome targeting (Pan et al., 2006). These degrons are utilized differentially, as they are required for the destruction of Gli1, for either the processing or destruction of Gli2, and for the processing of Gli3 (Bhatia et al., 2006; Huntzicker et al., 2006; Pan et al., 2006). Additional degrons are present in Gli1, which might utilize the Numb-Itch ubiquitination pathway or some other unidentified mechanisms of degradation (Di Marcotullio et al., 2006).

Suppressor of Fused (Sufu) is a key negative regulator of the Hedgehog pathway that functions downstream of Smo. Sufu interacts with all three Gli proteins, and sequesters Gli1 and Gli2 in the cytoplasm (Dunaeva et al., 2003; Kogerman et al., 1999), or inhibits Gli transcriptional activation via recruitment of sin3-associated polypeptide 18 (SAP18) and the histone deacetylation machinery (Barnfield et al., 2005). Sufu also promotes the proteolytic cleavage of Gli3 to form the Gli3 repressor (Kise et al., 2009) or interacts with full length Gli3 (Gli3FL) in the absence of Shh signaling (Humke et al., 2010).

### 2.3.2 Role of Hh in ectodermal organ development

*Shh* expression has been detected both in tooth and hair placode epithelium but is barely detectable in the mammary gland placode (Bitgood and McMahon, 1995). In later stages of tooth development, *Shh* is expressed in the enamel knot and ameloblasts (Koyama et al., 1996). *Shh* null embryos die before birth (Chiang et al., 1996); therefore, genetic studies on tooth development have been made using conditional knockouts such as *Smo* deletion in the dental epithelium. This study showed the importance of Shh signaling in regulating tooth morphogenesis, as the first and second molar were fused in *Smo* transgenic mouse. Furthermore, the cusps were underdeveloped and misshapen (Gritli-Linde et al., 2002). Genetic studies showed that repression of Hh signaling is needed by certain mammary gland placodes to acquire the mammary gland fate, as in the *Gli3* null mouse where Hh inhibition is abrogated and is missing two mammary glands (Hatsell and Cowin, 2006).

### 2.4 Fibroblast growth factor pathway

Fibroblast growth factors (FGFs) are polypeptide growth factors with diverse biological activities during development, repair, and metabolism and they regulate a variety of cellular processes such as differentiation, proliferation, and migration. *Fgf1* and *Fgf2* were first purified in 1975 from bovine pituitary gland (Gospodarowicz and Handley, 1975). Since then, 22 members have been identified and classified according to their mechanisms of action (*Fgf1-23*). *Fgf19* does not exist in mouse and rat, whereas *Fgf15* is absent from human. These Fgfs can be classified as canonical Fgfs, intracellular Fgfs (iFGFs), and hormone-like Fgfs (hFGFs) and can employ intracrine, paracrine
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and endocrine signaling, respectively (Itoh, 2010; Itoh and Ornitz, 2008). Paracrine or canonical Fgfs bind and activate Fgf receptors (Fgfrs), cell surface tyrosine kinase proteins, to mediate the corresponding biological response (Beenken and Mohammadi, 2009).

Paracrine FGFs comprise Fgf1-10, Fgf16-18, Fgf20 and Fgf22. Most are secreted proteins with cleavable N-terminal signal peptides; however, Fgf9, Fgf16 and Fgf20 have uncleaved bipartite secreted signal sequences (Koga et al., 1999; Miyake et al., 1998; Revest et al., 2000). On the contrary, Fgf1 and Fgf2 are not secreted proteins but seem to be released from damaged cells or by exocytosis (Mohan et al., 2010; Nickel, 2010).

2.4.1 FGF canonical signaling pathway

Fgf ligand proteins exhibit a heparan sulfate glycosaminoglycan (HSGAG) binding site (HBS) in their structure. Paracrine Fgfs induce their signal by binding to and activating Fgfr in an HSGAG-dependent manner (Itoh and Ornitz, 2011). Four Fgfr genes (Fgfr1-Fgfr4) have been identified and give rise to seven tyrosine kinase receptor proteins by alternative splicing and exon skipping: (Fgfr1b, 1c, 2b, 2c, 3b, 3c and 4). Each of these proteins have different ligand binding affinities and are predominantly epithelial or mesenchymal Fgfrs (Beenken and Mohammadi, 2009; Itoh and Ornitz, 2011; Turner and Grose, 2010). A functional FGF-FGFR unit consists of two 1:1:1 FGF-FGFR-HSGAG complexes juxtaposed in a symmetrical dimer. Each ligand binds to both receptors and the receptors contact each other (Beeken and Mohammadi, 2009). During development, paracrine Fgfs seem to influence the intracellular signaling of neighboring cells. The range of action is determined by the affinity of the ligand for extracellular matrix heparan sulfate proteoglycans (HSP) and the equilibrium between homo- and dimerization of the ligand (Kalinina et al., 2009). Fgf binding to Fgfrs induces receptor transphosphorylation to activate four key downstream signaling pathways: Phospholipase C-gamma (PLCγ), which stimulate the release of intracellular Ca^{2+}; RAS-RAF-mitogen-activated protein kinase (MAPK); phosphatidylinositol 3-kinase/AKT (PI3K/AKT); and the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathways. All three of these pathways are implicated in cell cycle, survival, migration and differentiation (Umbhauer et al., 2000; Bottcher and Niehrs, 2005; Itoh and Ornitz, 2011; Turner and Grose, 2010).

2.4.2 Role of FGFs in ectodermal organ development

Most Fgf genes have been disrupted by homologous recombination in mice. During early tooth development, Fgf8 has been proposed to compete with Bmp2/4 in order to commit cells from the mandibular arch to induce tooth development (Neubuser et al., 1997). When tooth placode is initiated, Fgfr2b regulates placode downgrowth as Fgfr2b-null tooth development is arrested at the bud stage (Celli et al., 1998). Fgf3 and Fgf4 seem important for tooth cusp formation hence tooth morphogenesis (Charles et al., 2009; Jernvall et al., 1994; Kettunen and Thesleff, 1998). During mammary gland development, Fgfr2b is expressed in the mammary epithelium, whereas its ligand, Fgf10 is expressed in the lateral reaches of somites close to the future mammary placode site.
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(Mailleux et al., 2002). FgfR2b and Fgf10 have an important role in mammary gland induction as four mammary buds (number 1,2,3,5) fail to develop in both Fgfr2b and Fgf10 mouse mutants (Mailleux et al., 2002). Interestingly, in the Fgfr2b null mouse, the remaining mammary bud number 4 undergoes apoptosis after initiation and is therefore not maintained (Mailleux et al., 2002).

3 The TNF superfamily

The TNF superfamily is composed of 18 genes giving rise to 19 protein ligands. The TNF receptors (TNFR) superfamily comprises 29 members in human and 27 in mouse (Figure 6) (Bodmer et al., 2002; Bossen et al., 2006).

The TNF superfamily of ligands are type II transmembrane proteins, which mean that their N-terminus is intracellular. TNFs are active either as membrane bound or soluble proteins generated by proteolytic cleavage. TNF receptors (TNFR) are characterized by up to six Cysteine Rich Domains (CRDs). For example, B-cell activating factor receptor (BAFFR) has one CRD, whereas TNFRI and TNFRII have three (Figure 6). They usually form trimeric structures in order to convey their signal. Upon receptor stimulation, some TNFR superfamily members are cleaved from the cell surface, such as TNFRII and 4-1BB, or directly expressed as soluble forms if they are lacking the transmembrane domain (Bossen et al., 2006; Idriss and Naismith, 2000).

Upon ligand activation, TNFRs recruit several intracellular adaptor proteins, which in turn activate different signal transduction pathways. Based on the adaptors TNFR recruits, the TNFR superfamily members are classified into three different categories: 1) TNFR proteins containing a death domain (DD) in their cytoplasmic domain; 2) TNFRs lacking a death domain but having TNF receptor associated factor (Traf) interacting motifs; and 3) TNFRs that do not contain either of these domains (Figure 6) (Dempsey et al., 2003).

The first group (Figure 6) includes FAS (APO-1, CD95), TNFRI (p55), p75 (NGFR), and Ectodysplasin Receptor (Edar) (Dempsey et al., 2003). The TNF receptor family member p75 is unique in that it binds neurotrophins that do not belong to the TNF family. DcR2 possesses a truncated DD, which does not allow transmitting any signal; however, DcR2 is involved in inhibiting DR4/DR5 signaling in human (Almasan and Ashkenazi, 2003; Yano and Chao, 2000).

The second group of receptors does not contain any DD but TRAF interacting motif(s) (TIM) in their intracellular domains (Figure 6). This group includes Lymphotoxin-β receptor (LtβR), Receptor activator of NFκB (Rank), Glucocorticoid-induced TNF-receptor (Gitr), X-linked EdaA2 receptor (Xedar), and Troy. Only six TRAF proteins have been identified (Traf1-6) (Grech et al., 2000). Although they do not seem to have any enzymatic activity, they are able to induce pathways involved in many cellular processes such as cell proliferation, differentiation, and cell death.

The third group of TNFR includes only Trail-R3 (DcR1), DcR3, and Osteoprotegerin (OPG). They are secreted receptors that seem to compete with the receptors from the two other groups (Figure 6) (Dempsey et al., 2003).
Figure 6. The TNF/TNFR superfamily. The TNF homology domain of the ligands are shown in pink and arrows indicate interaction with their receptors. The ectodomains of the TNFR superfamily are shown in green with their number of CRDs. Some of the receptors ligands are not known (?) or not part of the TNF superfamily (*). Receptor names in smaller font (DcR3 and DcR2) are not found in mouse. Receptor names in blue indicates the presence of a death domain, names in black highlight receptors with Traf interacting domain(s), and names in green represent receptors with no transmembrane domains.
TNF family of ligands can bind to one or more receptors. However, four receptors are considered orphans as their identified ligands do not belong to the TNF superfamily or are not known namely: p75, DR6, Troy, and Relt (Bossen et al., 2006).

3.1 The death receptor signal transduction pathway and the caspase cascade

As a common rule, activation of death receptors (DRs) induce the recruitment of adaptor molecules that also contain a death domain such as Fas-associated death domain (FADD) or TNFR-associated death domain (TRADD) (Ashkenazi and Dixit, 1998). These proteins contain one DD that will interact with the death receptor DD and one death effector domain (DED) that enables interactions with other DED bearing proteins such as procaspases. The most extensively studied death receptors are Fas, TNF-R1, DR4, DR5, and the recently emerged DR6 (Figure 6) (Nikolaev et al., 2009; Wajant, 2003).

DRs can be divided into two categories based on the primary adaptor protein they bind to: Fas, DR4, and DR5 bind FADD directly and mostly have proapoptotic functions. Cell apoptosis by TNFRs proceed through three general steps. The first step involves the binding of the ligand on its death receptor. The second step is the recruitment of adaptor/docking proteins FADD by the DD, which, in turn, recruit the initiator caspases 8 (or caspase 10 in human cells) by interaction between DED contained in FADD and the prodomain of both caspases (Nikolaev et al., 2009; Scaffidi et al., 1999; Yang et al., 2010). The complex thus formed is called death-inducing signaling complex (DISC). The third step induces the cleavage of executioner procaspases directly and leads to apoptosis of the cell (Figure 7) (Gonzalvez and Ashkenazi, 2010; Wilson et al., 2009).

TnfRI is an example of the other category as it can trigger apoptosis through protein serine-threonine kinase 1 (RIP1) or TRADD-dependant recruitment of FADD and caspase 8 or 10 (Wang et al., 2008). Tweak also seems to induce caspase pathway using FADD and RIP1 (Ikner and Ashkenazi, 2011).

The TNF pathway can make life or death decisions for the cells; therefore, it is important to tightly regulate its activation. This regulation is assured at numerous levels including the regulation of ligand/receptor expression, soluble decoy receptor expression, and anti-apoptotic ligand induction. One of these checkpoints is set by the protein silencer of DD (SODD), which can bind to TNFRI and DR3, and seems to prevent ligand-independent activation of certain death domains. SODD binds to the death domain of these receptors, thus inhibiting TRADD recruitment until ligand binding disrupts SODD binding and allows TRADD to take its place (Jiang et al., 1999; Walczak, 2011).

Activation of caspase 8 or 10 can be disrupted by cellular FLICE-inhibitory (cFLIP) proteins. Three cFLIP isoforms are known and all contain DEDs that mediate recruitment to the DISC and all three isoform are able to prevent cell apoptosis. However, the long cFLIP isoform seems to also be able to promote or prevent apoptosis depending on the death ligand engaging the caspase cascade (Asaoka et al., 2011).
Inhibitors of apoptosis (IAPs) also help controlling the induction of cell apoptosis by specifically inactivating effector caspases (Suzuki et al., 2001; Uren et al., 1996).

Interestingly, TNFRI does not primarily induce apoptosis, but more likely the activation of the Nuclear factor-κB (NFκB) and transcription of its target genes, or the cJun N-terminal kinase (JNK) pathway that affects the actin cytoskeleton, and therefore cell polarization and migration (Mathew et al., 2009). TNFRI only signals death when protein synthesis is blocked or when the NFκB pathway cannot be induced, such as when a mouse mutant does not have the RIP gene (Hsu et al., 1996; Kelliher et al., 1998).

**Figure 7.** Pathways induced by the TNF family members.
3.2 TNFR signaling pathway through TIM

Activation of TIM containing TNFR family members directly recruit Traf proteins leading to the activation of pathways like NFκB, JNK, p38, Extracellular signal related kinase (ERK), and PI3K/AKT (Dempsey et al., 2003; Ha et al., 2009). The most studied Trafs are Traf2, 5, and 6 for their involvement in NFκB pathway activation and ectodermal development. It is suspected that Traf2 also influences the non-canonical NFκB pathway upon activation by LtβR, CD40, and BAFFR by interacting with NFκB inducing kinase (NIK) (Kanno et al., 2010; Malinin et al., 1997; Qing et al., 2005). RANK seems to activate NFκB pathway using Traf 2, 5, and 6 (Darnay et al., 1998; Darnay et al., 1999). Traf3 has also been implicated in non-canonical NFκB activation, most likely following CD40 or BAFFR activation (Hacker et al., 2011).

Traf6 does not only interact with TNFRs but also with TGFβ receptors and seems to induce its own signal transduction via binding to TGFβ activated kinase-1 (TAK1) to induce not only the JNK pathway but also the canonical NFκB pathway (Deng et al., 2000; Landstrom, 2010).

3.3 The main TNFR signaling pathway: NFκB

All TNFRs can signal through NFκB. Also the NFκB signaling pathway plays a role in many of the biological functions mediated by TNFR family members and most importantly, Edar (Figure 7) (Hehlgens and Pfeffer, 2005; Maksimow et al., 2006).

The mammalian NFκB family comprises five members: p65 (RelA), RelB, c-Rel, NFκB1 (p50 and its precursor p105), and NFκB2 (p52 and its precursor p100). p105 and p100 are processed post-transcriptionally into p50 and p52, respectively. Both p50 and p52 subunit show a Rel-homology domain (RHD) which contains a nuclear localization sequence and is involved in dimerization with other Rel, sequence specific DNA-binding, and interaction with their inhibitory IκB protein (Ghosh et al., 1998). NFκB proteins are present in unstimulated cells as homo- or heterodimers (e.g p50/p50 or RelA/p50). Interestingly, p50 or p52 homodimers function as repressors of transcription while dimers that contain RelA or c-Rel are transcriptional activators (Ghosh et al, 1998). RelB heterodimers can act both as activators and inhibitors (Beg et al., 1992).

Processing of p100 or p105 does not lead to the transduction of the same pathway: if precursor is p105 is processed, it activates the canonical NFκB target genes transcription and if p100 is processed, then the non-canonical pathway genes will be induced (Silke, 2011). Whether or not these genes are different is not actually known as downstream target genes of both pathways are still being discovered. The non-canonical NFκB pathway can be induced only by a subset of TNFR such as LTβR, CD40, CD27, BAFFR, RANK and Tweak in vivo (Razani et al., 2011).

Canonical NFκB pathway

The canonical NFκB pathway involves mostly the heterodimer p50/relA and is preferred by almost all TNF family members.

In unstimulated cells, NFκB dimers are kept inactive by binding to members of the IκB family (most commonly IκBα) via multiple ankyrin repeats (Moynagh,
2005). Activation of the NFκB pathway depends on IκB degradation, which occurs after phosphorylation on two key serines by IκB kinases (IKKs) that form a complex (Yamamoto and Gaynor, 2004). The IKK complex is composed of two IκB kinases: IKKα and IKKβ that form the catalytic subunits and a scaffolding subunit IKKγ (also called NEMO) that is responsible for activating the NFκB canonical pathway (Rahighi et al., 2009; Tokunaga et al., 2009). Phosphorylation of IκB proteins precedes the binding of E3 ubiquitin ligase complex β-TrCP-SCF, which then polyubiquitinates IκB proteins and targets them for proteasome destruction (Karin and Ben-Neriah, 2000). Once IκB proteins are degraded, NFκB will translocate into the nucleus in order to induce the transcription of its target genes (Figure 7) (Tergaonkar et al., 2005).

It was also shown that TNFRI also recruits Traf2 upon binding of RIP1 to TRADD and hence induces NFκB activation of the caspase cascade (Figure 7) (Gentle and Silke, 2011).

There are two major antagonists of NFκB signaling upon TNF activation: A20 and CYLD. A20, also known as Tnfaip3 (for TNF-associated inducible protein 3), is a zinc finger protein able to act as a feedback inhibitor responsible for the arrest of NFκB signaling after Tnfα signal induction. Cylindromatosis (CYLD) is a deubiquitinylating enzyme that inhibits TRAF mediated ubiquitinylation and activation of NFκB (Harhaj and Dixit, 2011; Kovalenko et al., 2003; Shembade et al., 2010). It was recently suggested by Ting A.T that NEMO is also able to block caspase signaling by directly binding to RIP1, thus abrogating caspase 8 recruitment before NFκB induces the transcription of anti-apoptotic genes (Eriksson and Vandenabeele, 2011).

3.4 Implication of TNF signaling in mediating cell shape changes

Studies postulated that TNFα modulates cytoskeleton conformation through the regulation of Rho-family GTPases. The Rho-family GTPases Cdc42, Rac, and Rho are central to many signaling pathway that leads to cytoskeletal changes. In endothelial cell culture, induction of TNFα seems to increase F-actin levels, stress fiber formation, and cell contraction via a hierarchical activation of Cdc42, Rac, and Rho (Mathew et al., 2009). Despite many studies pointing to the Rho-family of molecule as a downstream target of TNFα, the exact pathway(s) implicated remains elusive. It is postulated that TNFα does not require its usual adaptor such as Traf2 to induce such signaling (Puls et al., 1999). Therefore, JNK and NFκB pathways do not appear to be involved in cytoskeletal changes, but rather MAPK, PI3K or PLCγ1 and Ceramide (Hanna et al., 2001; Kutsuna et al., 2004; Papakonstanti and Stournaras, 2004).

Some TNFR such as Troy and p75 seem to play a role in axonal growth cone collapse which depends on RhoA-dependent changes in the cytoskeleton (Gallo and Letourneau, 2004). p75 has been shown to regulate RhoA activity in such processes by binding its ligand Nerve Growth Factor (NGF), which reduces RhoA activity (Park et al., 2006).

TNF signaling also seems to affect components of the cell-adhesion complexes, which mediate adhesive contacts between cells and their environment. For example, TNFα treatment can increase the tyrosine phosphorylation of adherens-junction such as -catenins and integrins, and this modification is mediated by either the Src-family kinases.
(Angelini et al., 2006; Bouaouina et al., 2004) or by the JNK pathway (Nwariaku et al., 2004). For example, in endothelial cells, TNF-mediated modulation of tight junction formation is correlated with changes in cell shape (McKenzie and Ridley, 2007).

3.5 Role of TNFRs during ectodermal organ development

Three TNFRs and two TNF ligands have been involved with ectodermal development so far namely Edar, Troy, TNFRI, Rank, and its ligand Rankl and lymphotokinβ (Ltβ). Rank has been also associated with the formation of lymph nodes and osteoclastogenesis (Walsh and Choi, 2003; Yoshida et al., 2002). Both Rank and Rankl deficient mice show osteopetrosis and no tooth eruption (Kong et al., 1999) due to the lack of co-ordination between odontogenesis and the underlying bone formation (Ohazama et al., 2004). Rank and Rankl are also involved in lobulo-alveolar development of mammary glands during pregnancy (Fata et al., 2000). Other TNFs and TNFRs are involved in hair development.

Edar and Troy are the most structurally closely related receptors with 33% homology in their extracellular domain (Kojima et al., 2000) and as revealed earlier they both induce NFKB pathway activation. Troy and Edar are part of two different TNFR groups as Troy has an intracellular TIM allowing the binding of TRAF2 and the activation of NFKB pathway (Hu et al., 1999; Kojima et al., 2000). Troy also induces the JNK pathway using its cytoplasmic domain (Hu et al., 1999) and can promote apoptosis although it does not seem to require caspase involvement but another unknown mechanism (Eby et al., 2000). Troy has long been considered as an orphan receptor as interaction studies have shown that it does not bind to any TNF ligand in vitro (Bossen et al., 2006). However, one study showed that co-transfection of Troy with Lta activated NFKB, thus Lta could be the ligand for Troy (Hashimoto et al., 2008).

4 EdaA1 pathway

4.1 Ectodysplasin

The Ectodysplasin (Eda) gene is the most studied TNF family member during ectodermal organ development. The EDA gene was first cloned in human in 1996 by Kere and colleagues (Kere et al., 1996). Once cloned in the mouse mutant for Eda (Srivastava et al., 1997), it was found to belong to the TNF superfamily of ligands (Copley, 1999; Ezer et al., 1999; Mikkola et al., 1999). There is high amino acid conservation between human and murine Eda proteins with an overall homology of 95% for both proteins and 100% for the TNF domains.

The EDA gene spans approximately 425 kb of genomic sequence on the human X chromosome and encodes ectodysplasin A. A total of eight isoforms can be created by alternative splicing of the 12 exons. However, only two transcripts: Ectodysplasin A1 (EdaA1) and Ectodysplasin A2 (EdaA2) have been shown to have a biological function (Bayes et al., 1998; Cui and Schlessinger, 2006; Mikkola and Thesleff, 2003). EdaA1 is the longest transcript encoding a 391 amino acid protein. EdaA1 comprises a furine cleavage site, a collagen-like domain consisting of 19 Gly-X-Y repeats with
a single interruption, an HSPG-binding region, and a TNF homology domain (Bayes et al., 1998; Monreal et al., 1999; Srivastava et al., 1997; Swee et al., 2009). In order to bind to its receptor Edar, EdaA1 has to be released by proteolytic processing at the furin recognition site (Chen et al., 2001; Schneider et al., 2001). Further multimerisation through the collagen-like region has been shown and seems to be required for EdaA1 function in vivo (Schneider et al., 2001; Swee et al., 2009). Interestingly, the distribution of EdaA1 protein is also regulated by its HSPG binding region, which may act as a modulator of Eda activity but also helps stabilize the protein (Swee et al., 2009).

EdaA2 differs from EdaA1 by a two amino acid deletion due to alternative splicing. Interestingly, EdaA1 and EdaA2 do not bind to the same receptor, as EdaA2 uses the TNF receptor Xedar exclusively (Yan et al., 2000; Bossen et al., 2006). Structural studies of the receptor binding domains of EdaA1 and EdaA2 showed only 30% identity and the crystal structures revealed differences in electrostatic properties and protein surfaces, thereby giving an explanation for the different receptor affinities (Hymowitz et al., 2003; Yan et al., 2000). So far, no role for EdaA2 during ectodermal organ development has been found (Gaide and Schneider, 2003; Mustonen et al., 2003; Newton et al., 2004). Therefore, I will mainly focus on the role of EdaA1 during hair and other ectodermal organ development and will from now on refer to EdaA1 as Eda.

4.2 Signal transduction

Binding of Eda on its receptor induces the trimerization of Edar. Edar belongs to the first class of TNFR superfamily (Figure 6), which means that Edar protein has a cytoplasmic death domain able to bind a specific death domain adapter protein called Edaradd (Headon et al., 2001; Yan et al., 2002). In turn, Edaradd recruits a Traf protein (Traf6 in vivo), which binds TAK1-binding protein 2 (Tab2) that links Traf6 to TGFβ–activated kinase (TAK1) (Figure 8) (Morlon et al., 2005; Naito et al., 2002). This association allows the activation and degradation of the IKK complex and canonical NFκB pathway activation (Figure 8) (Koppinen et al., 2001; Headon et al., 2001; Yan et al., 2000; Zonana et al., 2000).

NFκB transcription factor activation is a key step for ectodermal organ development (Mikkola and Thesleff 2003). Furthermore, studies of the NFκB reporter mouse revealed that NFκB is active in many ectodermal organs such as hair, mammary gland, and tooth (Dickson et al., 2004). Interestingly, NFκB activity in ectodermal organs was lost in the Eda null mouse and increased when EdaA1 cDNA was overexpressed in the epithelium during development (Schmidt Ullrich et al., 2006; Pispa et al., 2008; Mikkola 2007).

4.3 Eda pathway and HED in human

As mentioned previously, mutation of EDA in human gives rise to X-linked hypohidrotic ectodermal dysplasia (HED) (XLHED; OMIM305100), a disease that affects ectodermal organ formation. Here I present the characterization of this disease in human and the impact of other Eda pathway members in HED.
Figure 8. Eda signaling. EdaA1 binds to its receptor Edar, which in turn binds to an adaptor molecule (Edaradd) through their death domains. Edaradd recruits Traf6, which in turn binds to TAK1 binding protein 2 (TAB2) and Tgfβ activated kinase 1 (TAK1). TAK1 then activates the IκB kinase (IKK) complex, which in turn phosphorylates IκB and target it to proteasome destruction, leaving NFκB free to translocate into the nucleus and induce the transcription of its target genes. Proteins with their names in red give rise to HED when mutated.


4.3.1 EDA and XL-HED

A famous early description of this disease was made by Charles Darwin who wrote about the toothless men of Seinde (India). Darwin described the men of this village as showing very little hair on the body, only ten misshapen teeth in both jaws, and suffering much during hot weather from dryness of the skin (Darwin, 1875). Darwin noticed that the disease affected only male members of one family and left the females unaffected. However, the daughters of affected males transmitted the disease to their sons but the affected males never did so. This particularity was later characterized as a recessive mutation on the X-chromosome (Hutt, 1935; Kerr et al., 1966; Kere et al., 1996).

Later reports highlighted that the hypohidrosis or anhidrosis was a consequence of sweat gland malfunction or absence (Clarke, 1987; Clouston, 1929) causing significant morbidity and mortality in affected children due to hyperthermia from their inability to sweat (Clarke et al., 1987; Gilgenkrantz et al., 1989). However, it seems that nowadays early recognition of the disease coupled with adequate instruction of the parents can greatly reduce the risk of morbidity and mortality during infancy (Bluschke et al., 2010). Tooth abnormalities include delayed primary and secondary tooth eruption, conical tooth crowns, and missing teeth (oligodontia), which lead to difficulties with mastication, poor appearance, and speech impairment (Johnson et al., 2002; Tape and Tye, 1995). The hypotrichosis (few hair) includes sparse scalp hair that fall off early in life, missing or scanty eyebrows and lashes, and overall reduced body hairs (except pubic and beard hair) (Clarke et al., 1987). Over 30% of patients also have breast abnormalities and one case study reported a complete absence of breast (Clarke, 1987; Megarbane et al., 2008; Smith, 1929). Taken together, hypohidrosis or anhidrosis, hypodontia and hypotrichosis are all symptoms defining HED (Clarke et al., 1987; Pinheiro and Freire-Maia, 1994; Reed et al., 1970; Smith, 1929). Analyses of EDA mutation in HED patients showed that mutation either in the furin cleavage or the collagen-like or TNF-binding domain, give rise to HED (Cui and Schlessinger, 2006; Mikkola and Thesleff, 2003; Schneider et al., 2001).

Recent reports about X-linked non-syndromic hypodontia described hypomorphic mutations in Eda, which means that diminished Eda signaling affects mainly tooth development (Mues et al., 2009; Schneider et al., 2001; Zhang et al., 2011).

4.3.2 EDAR, EDARADD and HED

In patients with HED, more than 100 different mutations in EDA, approximately 30 in EDAR, and a couple in EDARADD have been reported (The Human Gene Mutation Database: http://www.hgmd.cf.ac.uk). However, as the EDAR gene is present on autosomal chromosome 2 and EDARADD on chromosome 1, the genetic mode of transmission is different (Headon et al., 2001; Lamartine, 2003; Monreal et al., 1999). Mutations in EDAR account for one quarter of non X-linked HED, whereas mutations in EDARADD have proven to be rare (Chassaing et al., 2006; Chassaing et al., 2010). EDAR mutations usually give rise to either autosomal recessive transmission of the disease (Bashyam et al., 2011; Chassaing et al., 2006; Naqvi et al., 2011), although
autosomal dominant transmission has also been reported (Lind et al., 2006). EDARADD mutations only account for few autosomal recessive or dominant HEDs (Bal et al., 2007; Chassaing et al., 2010).

### 4.3.3 Mutations of downstream effectors

HED has also been found in association with other defects associated with immune deficiency. Mapping of genes responsible for these forms of HED showed that downstream players of Eda pathways were involved.

Hypomorphic mutations of NEMO (IkBγ), located on chromosome X, are usually responsible for XL-HED with immunodeficiency (ID) (Courtois et al., 2001; Zonana et al., 2000). Patients with XL-HED-ID (MIM 200248) show hypohidrosis, sparse hair, and oligodontia but also immunodeficiency, specific polysaccharid antibody deficiency, and elevated serum IgM levels (Smahi et al., 2002; Smahi et al., 2000). Stop codon mutations of NEMO cause a more severe syndrome associated with osteopetrosis and/or lymphoedema with HED-ID (OL-HED-ID, MIM 300301) (Dupuis-Girod et al., 2002). Males affected with XL-HED-ID or OL-HED-ID only suffer from hypomorphic mutations, which means that NFκB signaling is just diminished (Smahi et al., 2002).

Amorphic mutation of NEMO leads to another syndrome called incontinentia pigmenti (IP, MIM 304300), a rare X-linked dominant genodermatosis. Affected patients have Blaschko linear skin lesions (Landy and Donnai, 1993) associated with HED and anomalies in the nervous system (frequent seizure). In this syndrome NEMO expression is completely abolished and cells from these patients are unable to induce NFκB responses. However, symptoms of IP vary due to a skewed X-chromosome inactivation in certain tissues or within cell lineages where the normal counterpart is functionally important (Harris et al., 1992). Therefore, the mutated allele can be selected against and induce normal NFκB signaling in some cell lineages. This explains why IP syndromes are mostly sporadic. However, IP patient cells with a mutation in NEMO are highly sensitive to TNF-induced apoptosis, which could account for the development of IP lesions (Smahi et al., 2002).

A case report in 2003 stated that a mutation in IkBα caused HED with severe T-cell immunodeficiency. This autosomal dominant form of HED-ID is due to impaired but not abolished IkBα degradation resulting in impaired NFκB signaling (Courtois et al., 2003).

Recently, a woman was reported with a de novo mutation in TRAF6 that induced all the phenotypic features of HED (Wisniewski and Trzeciak, 2012). However, functional studies of the mutated protein were not conducted.

### 4.4 Eda pathway and HED in mouse

Many of the mouse models used to study the role of Eda pathway during development are spontaneous mutations and like in human, the mouse phenotype was studied long before the responsible gene was cloned. In this chapter, I aim to describe Tabby, the Eda null mutant model for XL-HED, as well as Edar and Edaradd null phenotypes.
4.4.1 XL-HED in mouse: Tabby

The Tabby phenotype, which corresponds to human XL-HED, was the first sex-linked phenotype described in mouse (Falconer, 1952). The mouse phenotype has since been extensively characterized by us and others. The Tabby hair coat contains only one type of Awl-like hair in contrast to wild type (WT) mice and shows abnormalities in the hair medulla (Gruneberg, 1966b). They usually have a hairless tail with a kink at the tip, fewer sensory vibrissae, and also a patch of hairless skin behind the ears where zigzag hairs reside in WT mice (Falconer, 1952; Gruneberg, 1966b). This hair phenotype can partially be explained by the delay in hair follicle development seen in Tabby mice. In fact, no hair placode is developing before E16 (Hardy and Vielkind, 1996), which means that the first wave of hair follicle formation that takes place around E14 in wild type (WT) mice and gives rise to guard hair in the adult is not induced in Tabby mice (Laurikkala et al., 2002).

Tabby mice also have a tooth phenotype as the third molars and the incisors are occasionally missing with variable penetrance depending on the mouse strain studied (Gruneberg, 1966a; Kristenova et al., 2002; Pispa et al., 1999). The developing first and second molars are reduced in size and their cusp size and number are also reduced (Grüneberg, 1966a; Pispa et al., 1999). This phenotype can be explained by the abnormal tooth development in Eda null mice, in fact, one day after the tooth placode has been induced, the Tabby molar placode looks smaller than in WT and later genes normally expressed in the enamel knot are marking a smaller area, meaning that the Tabby enamel knot is smaller than WT (Kangas et al., 2004; Laurikkala et al., 2001; Pispa et al., 1999). During the bell stage, the secondary enamel knots in Tabby fuse (Laurikkala et al., 2001; Pispa et al., 1999; Tucker et al., 2000).

The Tabby mouse also shows a defect in more than 20 glands. For example, sweat glands are missing from their foot pads and lacrimal glands are smaller and malfunctioning (Blecher et al., 1983; Gruneberg, 1971). Minor salivary glands located in the submucosa of the tongue, cheeks, and palate do not develop in Tabby (Wells et al., 2011). However, submandibular salivary glands do develop in Tabby but show decreased branching in in vitro culture (Haara et al., 2011). Also mammary glands show decreased branching in Tabby (Voutilainen et al., 2012).

4.4.2 HED in mouse: Edar and Edaradd mutations

In addition to Tabby, three other natural mouse mutants have been identified with HED symptoms: Downless, Crinkled and Sleek (Sofaer, 1969a, b). Downless and Crinkled are autosomal recessive mutations, whereas Sleek is dominant (Crocker and Cattanach, 1979). It was later confirmed by genetic studies that Downless and Sleek mice have two different mutations in Edar (Headon and Overbeek, 1999) and the Crinkled mouse has a mutation in Edaradd (Headon et al., 2001; Yan et al., 2002). Therefore, mutations in either the ligand, receptor, or co-activator molecule causes the same phenotype, hence proving the importance of the Eda pathway for ectodermal organ development.
4.4.3 Mutations of downstream effectors

Several transgenic mice have been generated to study the importance of the Eda/NFκB pathway. Schmidt-Ullrich and colleagues (2001) generated a transgenic mouse with IκBα stabilized protein (which does not allow IκBα to be targeted by the proteasome) under the promoter region of β-catenin: the cIkBAN transgenic mice. These mice show a loss of NFκB activity in ectodermal organs that leads to HED syndrome (Schmidt-Ullrich et al., 2001). Similarly, Ulvmar and colleagues showed that blocking NFκB signaling in the epithelium during development also gives rise to HED syndrome (Ulvmar et al., 2009). Taken together, these studies prove the importance of NFκB signaling in the epithelium during ectodermal organ development. Lack of Traf6 in mouse produces a skin appendage phenotype highly similar to the Eda null phenotype (Naito et al., 2002; Sinha et al., 2002), indicating that the Eda signal is transduced mainly by Traf6. Interestingly, TAK1 inhibition in keratinocytes leads to a defect in hair initiation and cycling (Sayama et al., 2010).

4.5 Role of Eda pathway in hair follicle development

Before the formation of hair placode, Eda is expressed throughout the epithelium. When hair placode formation is initiated at E14, Eda expression is localized to the interplacodal area. Concomitantly, the expression of its receptor will be restricted to the placodal epithelium where NFκB activity is also visualized; hence showing that Eda signaling occurs in the epithelium (Headon and Overbeek 1999; Laurikkala et al., 2002; Schmidt-Ullrich et al., 2006). It is not yet fully understood what regulates the Eda and Edar expression pattern in the skin in vivo. The Wnt signaling pathway seems to influence Eda expression. Especially, Wnt6 was shown to induce Eda expression in skin explant culture (Laurikkala et al., 2002). In fact, a Lef1 binding site was found 370 nucleotides upstream of the Eda transcription site and a promoter reporter study proved that this binding site could induce Eda expression in cell culture (Durmowicz et al., 2002). Therefore, Eda could be a direct target gene of the canonical Wnt pathway in vivo as well. However, when this promoter region was cloned and placed in front of a LacZ reporter gene it did not direct lacZ expression to ectodermal organs (Pispa, 2004).

As soon as the hair placode is formed, two TGFβ members seem to be involved in restricting Edar expression in the developing placode. ActivinβA acts as an inducer of Edar expression in the placode, whereas Bmp4 seems to surround the placode to restrict Edar expression (Laurikkala et al., 2002; Mou et al., 2006).

In the Eda null mouse, the first wave of hair placodes fails to initiate at E14. Furthermore, crossing of NFκB reporter mice with Tabby or Downless mice showed that the NFκB activity in these mice is lost at this stage, thus showing that NFκB activation is dependent on Eda pathway during placode formation (Pispa et al., 2008; Schmidt-Ullrich et al., 2006). Interestingly, most of the placodal markers expressed in the WT mouse do not show any localized expression at E14 in the Eda or Edar null mouse (Andl et al., 2002; Headon and Overbeek, 1999; Laurikkala et al., 2002). However, careful histological studies on Eda pathway mutant mice have revealed the existence
of rudimentary primary placode structures termed pre-placodes (Schmidt-Ullrich et al., 2006).

The expressions of Edar and Troy in ectodermal organ development are very similar (Kojima et al., 2000; Pispa et al., 2003). The double knockout for Eda and Troy does not show any sign of hair follicle formation until the third wave of hair follicle development around E18 (Pispa et al., 2008). However, in cκBAN transgenic mice with diminished NFκB signaling, the secondary hair follicles develop normally suggesting that although the Eda/NFκB pathway is important for the first wave of hair follicle formation, the second and third wave may require another additive signaling pathway downstream of Eda and Troy (Schmidt-Ullrich et al., 2001; Schmidt-Ullrich et al., 2006; Pispa et al., 2008). Troy can activate other pathways such as JNK (Eby et al., 2000), but the other pathway involved in the second wave of hair formation is yet to be discovered.

Generation of mice overexpressing EdaA1 cDNA in the ectodermal epithelium under the K14 promoter (K14-Eda) showed multiple defects in ectodermal organs including hair, mammary glands, and teeth (Mustonen et al., 2003). Two types of K14-Eda transgenic mice were generated using the same cDNA construct: K14-Eda with straight and curly hair. In both transgenic mice, the hair placodes that formed at E14 were increased in size and of irregular shape (Mustonen et al., 2004), giving rise in the adult to an increased proportion of guard-like hair (according to length comparison criteria performed on the K14-Eda straight) and all hairs presented a disturbed medullary structure (Mustonen et al., 2003). Surprisingly, hair placodes were produced continuously in the K14-Eda embryos; hence, new hair follicles were produced at E15, before the second wave of hair follicle formation that takes place at E16. The newly formed hair follicles were abnormally close to the previously formed ones and sometimes a fusion between hair follicles was observed especially in the K14-Eda curly mouse, an aspect never seen in the WT mouse (Mustonen et al., 2003). Also, transgenic mice expressing multiple copies of the entire Edar locus have larger primary hair placodes and a coarse coat in the adult (Mou et al., 2008).

Hairs of the K14-Eda mouse were overall longer than in WT due to a longer anagen phase during the first hair cycle (Mustonen et al., 2003). This result, taken together with the Edar null mouse showing accelerated catagen entry, indicates that the Eda pathway may control hair follicle maintenance of anagen by regulating cell apoptosis in keratinocytes (Fessing et al., 2006).

During hair placode downgrowth, the Eda pathway seems to induce Shh, the Bmp inhibitor connective tissue growth factor (CTGF), and probably indirectly CyclinD1 expression (Mou et al., 2006; Pummila et al., 2007; Schmidt-Ullrich et al., 2006).

5 Interplay of signaling pathways in hair formation

Our understanding of hair development and the importance of the above described pathways comes from studies on mouse mutants showing hair defects and ex vivo tissue cultures. In this chapter I will give an overview of the important interplay between pathways that will give rise to hair: from patterning of the smooth embryonic skin until the regeneration of hair in the adult.
5.1 Patterning

One key mathematical model in developmental biology was formulated by Alan M. Turing in 1952. This model called the “reaction-diffusion” model aimed at understanding how an ordered pattern could rise from initially homogeneous tissues (Turing, 1952). Turing proposed a simple model where two substances homogenously distributed initially would interact to produce stable pattern that would correspond to regional differences in the concentration of both substances (Figure 9). This model implies the interaction of two substances: an activator and an inhibitor and it hypothesizes that the activator is able to induce itself and its inhibitor, which in turn will inhibit the activator, hence the term reaction (Figure 9a). In this model, the activator does not diffuse as fast as its inhibitor in the tissue, hence the term diffusion. Therefore, the expression of the activator will increase in one location and be downregulated in the vicinity (Figure 9b).

**Figure 9.** The reaction diffusion (Turing) model. This model is based on an activator that is able to induce its inhibitor and its own transcription. The inhibitor diffuses faster than the activator in the tissue (a). The distribution of activator and inhibitor is initially random. As the activator concentration increases locally, it produces more inhibitor, which diffuses to inhibit other activator peak formation in the vicinity (b). This results in a series of peaks at regular intervals that resemble mouse hair placode formation during embryonic development (c reference state). However, by tampering with the concentration of activator or inhibitor, the inhibitor range of inhibition is increased (More inhibitor) or decreased (More activator) and the final hair placode pattern is affected.
The reaction-diffusion model predicts alternating areas of high and low concentration of the activator and when the activator reaches a certain threshold, the cells will start differentiating in a certain way (Figure 9b). Therefore, only certain high concentrations of activator will induce differentiation of the cell, whereas lower concentrations will be overruled by the inhibitor.

In 2006, Sick et al. used this mathematical model in mouse hair development to test if the canonical Wnt pathway and its inhibitor Dkk could account for hair follicle pattern formation in the mouse. They generated a mouse mutant overexpressing Dkk2. In this mutant, the over activation of the inhibitor changes the hair placode pattern which translates in the adult to sparser hair distribution (Figure 9c). Mou and colleagues (2006) studied the role of the Edar pathway in hair pattern formation but this issue will be discussed more extensively in the results and discussion chapter.

5.2 Placode formation

The Wnt pathway is one of the major regulators of hair placode formation, as inhibition of canonical pathway components using K14-Dkk1 leads to a complete blockade of hair follicle formation (Andl et al., 2002). Also, mice lacking Lef1 show an absence of the majority of coat hair follicles due to a 33% reduction of hair placode induction and a subsequent hair follicle morphogenesis arrest (van Genderen et al., 1994). Conditional deletion of β-catenin in mouse during embryonic development also affects hair development, as hair placode formation is abolished in this mutant (Huelsken et al., 2001; Zhang et al., 2009). Comparatively, stabilization of β-catenin in the mouse epithelium provoked precocious hair placode formation as early as E12.5 and the continuous induction of hair placodes afterwards (Narhi et al., 2008; Zhang et al., 2008), which corresponds to a previous study from Ouji et al., 2006 where administration of active Wnt10b on E10.5 epithelial skin induced precocious hair follicle formation two days later (Ouji et al., 2006). Stabilization of β-catenin in the epithelium can overcome the lack of Eda pathway activation, as Eda-/-; β-catenin\textsuperscript{stabilized}/WT mouse mutants are able to induce the first wave of hair follicle formation normally lacking in the Tabby mouse mutant (Narhi et al., 2008), thus suggesting that Wnt pathway signaling is downstream of Eda signaling and is necessary and sufficient to induce hair placode formation. On the other hand, in vivo studies indicated that Wnt/β-catenin signaling is active before Eda in skin and is required for activation of the Eda pathway at the onset of hair placode formation (Mou et al., 2006; Zhang et al., 2009). Taken together, these studies show that Wnt is upstream of the Eda pathway during placode initiation and later on, during placode formation, Wnt signaling and the Eda pathway act in parallel (Zhang et al., 2009; Narhi et al., 2008).

The TGFβ family and especially its Bmp members have been shown to have a critical role during placode formation (Millar, 2002). In vitro skin culture studies showed that hair placode formation was inhibited by Bmp4 protein. Similarly, adding a Bmp2/4 antagonist like Noggin stimulates hair placode formation (Botchkarev et al., 1999). Pummila and colleagues have shown that Noggin was able to restore hair placode formation in Eda null back skin (Pummila et al., 2007). Study of the Noggin null mouse showed that the number of hair placodes is decreased (Botchkarev et al., 1999;
Botchkarev et al., 2002) and grafting Noggin null skin on WT host showed that hair formation was not pursued in the adult (Botchkarev et al., 2002). Surprisingly, in this mutant the first wave of hair placode formation occurs normally, but the second wave is affected by the loss of Noggin (Botchkarev et al., 2002). Interestingly, Lef1 was shown to be induced in WT skin treated with Noggin. Hence, it seems that Bmp signaling needs to be inhibited in order to allow hair placode formation and the activation of Wnt/β-catenin signaling at the site of placode formation (Botchkarev et al., 1999).

Fgf signaling was also shown to be important during hair placode formation, as Fgfr2b mutant mice show reduced hair placode formation (Celli et al., 1998; Petiot et al., 2003; Revest et al., 2000). Fgfr2b is expressed in the epithelium of ectodermal placodes and is activated by four known ligands, Fgf1, Fgf3, Fgf7 and Fgf10. The latter two Fgfs are expressed predominantly in the mesenchyme underneath epithelium expressing Fgfr2b (Mason, 1994; Ornitz et al., 1996; Orr-Urtreger et al., 1993). However, a more recent study localized FgfR2b expression in the interplacodal region of the epithelium (Richardson et al., 2009). In vitro studies showed that addition of Fgf7 on E13.5 embryonic skin inhibited the formation of hair placodes and that FgfR2b inhibition is crucial for hair placode initiation and localization of placodal inducers such as Lef1 of Shh (Richardson et al., 2009).

5.3 Hair follicle downgrowth

Shh plays an important role in hair follicle down growth. It is expressed in the epithelial condensate of the hair placode and Shh null mice initiate hair formation but further down growth and differentiation is abrogated. Hence, mature hair follicles fail to appear (Chiang et al., 1999; St-Jacques et al., 1998). Ptc1 and Gli1 are expressed in the placodal epithelium and mesenchyme, thus showing that Shh signaling is active in both compartments of the hair follicle (Dahmane et al., 1997; Ghali et al., 1999; Platt et al., 1997). However, only Gli2-/- can phenocopy the Shh-/- hair phenotype, thus showing that Gli2 may be the main regulator of Shh signaling (Mill et al., 2003). Furthermore, an in vivo study showed that crossing a mouse expressing a constitutive Gli2 activator in the epithelium with a Shh-/- mouse was able to partially rescue the hair phenotype of the Shh-/- mouse. In fact, hair follicles were able to grow deeper and even showed follicular differentiation when Gli2 was activated. However, Gli2 activation only could not restore completely folliculogenesis in Shh-/- (Mill et al., 2003).

Hair downgrowth also seems to depend on two important molecules: activinA and follistatin. ActivinA is a signaling molecule expressed in the mesenchyme and in developing dermal papillae (Feijen et al., 1994). Follistatin is an inhibitor of activinA, which is expressed in the epithelium, the hair matrix cells, and ORS (Nakamura et al., 2003). This complementarity of expression seems to highlight a role in the epithelial-mesenchymal interaction during hair follicle development (Millar, 2002). Furthermore, studies of the activinA transgenic mouse, which overexpresses activinA in the epithelium, and follistatin null mouse showed a delay in hair development in vivo (Matzuk et al., 1995; Nakamura et al., 2003). Tissue cultures studies showed that while follistatin stimulates hair downgrowth, activinA inhibits this process. Therefore, it was proposed that activinA serves as a “brake” to stop further downgrowth of hair.
Follistatin, in turn, releases this inhibition by scavenging activinA hence, induces further downgrowth (Nakamura et al., 2003). Tgfβ2 is required for hair placode downgrowth as deletion of Tgfβ2 in mouse leads to delayed hair downgrowth but not initiation (Foitzik et al., 1999). Interestingly, Tgfβ2 seems to induce proliferation of the bud cells during hair development by inducing Wnt/β-catenin activation, which in turn downregulates E-cadherin and allows cells to proliferate (Jamora et al., 2005).

5.4 Differentiation of hair follicle compartments

From the bulbous peg stage onwards (Figure 2), cells in the hair follicle differentiate to form the seven different epithelial layers composing the mature hair follicle. Several lines of evidence suggest a role for Wnt signaling during this stage, as Wnt reporter activity is visualized in hair shaft precursor cells as they begin terminal differentiation (DasGupta and Fuchs, 1999). These cells also express Lef1 and Dsh2 (DasGupta and Fuchs 1999, Millar et al., 1999), and Wnt3 is visible in the adjacent cells (Millar et al., 1999). Wnt3 overexpression in mouse skin and hair follicle induces altered hair matrix keratinocyte differentiation leading to shorter hair formation (Millar et al., 1999). Lef1 binding sites have also been found in the promoter regions of many hair shaft keratin genes (Zhou et al., 1995). Also, stabilized β-catenin in epithelial cells showed that the Wnt/β-catenin pathway promotes hair shaft-like differentiation of ectodermal cells, as hair follicles failed to develop normally and instead formed an invagination where keratinized material was accumulated (Zhang et al., 2008).

Increasing evidence suggests that Bmp signaling is also involved in the control of cell differentiation during hair follicle development. Bmp2, Bmp4, and Bmpr1A are broadly expressed in the epithelial and mesenchymal cells of the developing hair bulb, whereas the expression of Noggin is restricted to the dermal papilla and follicular connective tissue sheath cells (Botchkarev et al., 1999; Kulessa et al., 2000). Study of a transgenic mouse expressing the Bmp inhibitor Noggin under the Msx2 promoter, thus directing noggin expression to the hair bulb, showed that Bmps regulate hair shaft differentiation (Kulessa et al., 2000). Similarly, in vivo epidermal deletion of BmpRIA in the adult caused a defect in postnatal hair follicle differentiation due to lack of differentiation of both IRS and hair shaft (Andl et al., 2004).

Molecular regulators of hair lineages are secreted by differentiating cells to form the different compartments of the hair follicle. For example, Bmps and Shh are involved in the formation of inner root sheath cells (Andl et al., 2004; Kobielak et al., 2007), Sox9 and Shh for outer root sheath (Gritli-Linde et al., 2007; Vidal et al., 2005), and Wnt/β-catenin, Bmps and Notch for the expression of certain hair shaft keratins and/or hair shaft development (Narhi et al., 2008; Zhang et al., 2008; Andl et al., 2004; Kobielak et al., 2003; Lin et al., 2000).

Lymphotoxinβ is expressed throughout the epithelium at the onset of the placode formation stage and later is restricted to the matrix region of the developing hair follicle (Browning and French, 2002; Cui et al., 2006). Curiously Ltβ knockout mice do not show any striking hair follicle development defects, but a hair shaft formation impairment that differs from the Eda null phenotype (Cui et al., 2006).
5.5 Hair cycling

Hair cycling has been studied in mouse as a very good tool to understand human hair cycling. Multiple molecular regulators have been identified using mouse mutants showing hair follicle cycling anomalies and by gene expression profiling of distinct murine hair cycle stages (Lin et al., 2004; Stenn and Paus, 2001). However, the molecular mechanisms that drive the hair cycle clock remains obscure (Paus and Foitzik, 2004). In this chapter I will give an overview of the importance of the above mentioned pathways in hair cycling and for a more detailed description about hair cycling I will direct you to reviews such as Hsu et al. (2011) and Shimomura and Christiano (2010).

Mapping of the gene responsible for the spontaneous mutation angora in mouse led to the identification of Fgf5 as a key regulator of the anagen-catagen transition. In this mouse, the entry in catagen is delayed for several days, which accounts for the increase in hair length (Hebert et al., 1994). Additionally, TGFβ1 induces catagen onset, as the TGFβ1 knockout mouse also displays a delayed entry into catagen (Foitzik et al., 2000). The molecular crosstalk between downstream effectors of TNFα signaling and keratin17 (K17) may be in part responsible for controlling catagen entry by regulating apoptosis (Tong and Coulombe 2006). Additionally, ActivinβA seems to play an important role in anagen-catagen transition as catagen is retarded in mice overexpressing Activin βA (Nakamura et al., 2003). TNFRI seems to have a role in hair follicle cycling, as the TNFRI null mouse shows a delayed entry in catagen, most likely due to a defect in apoptosis induction prior the catagen phase (Tong and Coulombe, 2006).

The telogen or resting phase corresponds to a phase where morphologically the hair is quiescent. However, telogen coincides with major changes in gene activity, as showed by time course gene expression profiling of different hair cycle stages (Lin et al., 2004). Hence, telogen is not as much a resting phase as a key stage of hair cycling control (Schneider et al., 2009). Telogen can be divided into two phases: a phase refractory to hair follicle growth characterized by upregulation of Bmp2/4 and a competent phase sensitive to anagen inducing factors characterized by the activation of Wnt/β-catenin genes and downregulation of Bmps (Plikus et al., 2008; Schneider et al., 2009). It has been shown that application of Noggin to telogen skin induces the transition to anagen (Botchkarev et al., 2001). Similarly, activation of Wnt/β-catenin in mouse during the telogen phase induces the entry of the hair follicle into the anagen phase (Van Mater et al., 2003). Transient activation of Shh also induces hair follicle anagen entry (Sato et al., 1999). One of the most important steps to start anagen is the activation of stem cells. Although the molecular details remain unclear, it is very likely that Bmp and Wnt/β-catenin genes are the main players to control the balance between the quiescence of stem cells in telogen and activation in anagen (Fuchs 2007). The quiescence of stem cells seems to require the activation of Bmp signaling and the inhibition of the Wnt pathway via Tcf3 and Dkks while stem cell activation requires the activation of the Wnt pathway and expression of Bmp inhibitor in the dermal papilla (Fuchs, 2007).

During anagen, the dermal papilla will progressively move away from the bulge and the epithelial stem cell will re-adopt quiescence. However, stem cell progeny in the hair matrix will maintain the Wnt/β-catenin pathway active throughout anagen (Van mater et
al., 2003). In the precortical hair matrix, the transient amplifying cells will stop dividing and start differentiating into the different lineage of hair follicle epithelium (Fuchs and Horsley, 2008; Schneider et al., 2009). Recently, Rank was shown to be expressed in the hair follicle germ, bulge stem cells of the adult hair follicle, and also the basal cell layer of the epidermis of mouse. A study of mouse lacking Rank showed that this TNF receptor is important for anagen re-entry after the first hair cycle is completed, as Rank null hair cycling stopped at telogen (Duheron et al., 2011).

6 The chemokine family

Chemokines are mostly involved in the regulation of migration of various cells in the body, hence their name which stands for chemotactic cytokines. Chemokine ligands are small peptides (8-14kDa) and their receptors are G-protein coupled receptors. They are involved in immunological response by mediating specific leukocyte recruitment from the circulation to tissue compartments during inflammatory episodes (Zlotnik and Yoshie, 2000). Recently, many other functions among which cell differentiation and division have also been reported (Rotondi et al., 2007).

The chemokines are divided into four different groups depending on the position of the two N-terminal conserved cysteine residues: CC, CXC (X meaning any amino acid between cysteines), CX3C, or C. The nomenclature of their receptor follows the same logic so that CXC ligands most likely bind to CXC chemokines receptors (Zlotnik and Yoshie, 2000). Based on their expression patterns, the chemokines can also be divided into two groups: the “Inflammatory chemokines” induced during the inflammation responses and the “Homeostatic chemokines” involved in a broader range of function such as lymphoid organogenesis and general organogenesis (Zlotnik et al., 2006).

The binding of a chemokine to its receptor activates a series of downstream effectors that facilitate internalization of the receptor and signal transduction (Zlotnik and Yoshie, 2000). This leads to two main responses: integrin activation, which causes adhesion of cells, and polarization of the actin cytoskeleton. The latter allows directional sensing, cell polarization, accumulation of small GTPases, Rac, Cdc42, and PI3K at the leading edge, resulting in actin polymerization, and F-actin formation. Taken together, these changes cause actomyosin contraction and tail retraction leading to cell migration (Rotondi et al., 2007). The binding of ligands to their receptors can also induce other signaling systems, e.g., the tyrosine kinase receptor and Jak/STAT pathways. The CXC-chemokine family is quite unique among chemokines because of their positive and negative activity on the control of angiogenesis.

6.1 cxcR3 pathway

The mouse cxcR3 receptor can bind three different ligands: cxcl9, cxcl10, and cxcl11 (Cole et al., 1998; Farber, 1997). cxcl10, cxcl11, and cxcl9 are all called Interferon gamma (IFNγ)-inducible CXC-proteins and seem to usually be induced by IFNγ protein at the site of infection (Lacotte et al., 2009). However, other factors (such as TNFα, IL1β, LPS and some viruses) are also able to induce the expression of those
chemokines during the inflammatory response (Muller et al., 2010; Shin et al., 2010). These three chemokines also show significant structural homology, being more similar to each other than any other CXC-chemokines (Clark-Lewis et al., 2003). Furthermore, all three chemokines share the ability to induce the directional migration of activated and memory T-cells (Garcia-Lopez et al., 2001; Moser and Loetscher, 2001). Hence, these chemokines are considered as belonging to their own small subfamily. Additional complexity occurs due to the promiscuous interaction of both cxcR3 and its ligands with other chemokines (e.g., cxcl4) or receptors (e.g., ccr3) ((de Jong et al., 2008; Loetscher et al., 2001; Rappert et al., 2002). However, in general, the biological impact of these interactions still needs clarification. Although the main function of the cxcR3 ligand is the stimulation of T-cell and NK cell chemotaxis, these chemokines also have a variety of non-chemotactic actions on T-cells, as well as other cells. These include, for example, stimulation and/or suppression of angiogenesis and defensin-like antibacterial activity (Muller et al., 2010; Romagnani et al., 2004).

One interesting example of cxcR3 pathway involvement in non-inflammatory processes is the role of the cxcR3 pathway during wound healing (Satish et al., 2003; Yates et al., 2007). Healing a wound is a really intricate process requiring fibroplasia to insure wound closure and epithelialisation. Fibroplasia is achieved by fibroblast migration and proliferation into granulation tissue followed by the sequential deposition of specific matrix components and finally contraction and remodeling in order to reconstruct the dermal layer. The epithelial barrier is reestablished also using proliferation and migration mechanisms, but from another type of cell: the basal keratinocytes, which will afterwards differentiate to reform the squamous epithelium. Studies demonstrate that these two processes are temporally linked (Satish et al., 2003; Yates et al., 2007). CXC-chemokines have been shown to be capable of inhibiting fibroblast motility by preventing the rear de-adhesion of cells (Shiraha et al., 1999), thereby converting movement to matrix contraction (Allen et al., 2002). cxcl10 seems to appear during this phase produced by neovascular endothelial cells (Engelhardt et al., 1998), whereas cxcl11 is produced by basal keratinocytes in human (Tensen et al., 1999). cxcR3 was shown to be expressed and function on mesenchymal cells (Cockwell et al., 2002; Shiraha et al., 1999). As these cxcR3 pathway members and their receptor were also found to inhibit endothelial cell proliferation and migration (Luster et al., 1995; Yates et al., 2007) and fibroblast migration (Shiraha et al., 1999). They were expected to also signal dermal maturation; therefore, cxcl10 and cxcl11 were both studied during wound healing processes and mainly cxcl11 was found to enhance keratinocyte migration and inhibit fibroblast motility during the wound healing process in vitro and in vivo (Yates et al., 2008). However, cxcl10 seems to also play this role but at lesser extent due to its lower affinity for the cxcR3 receptor compared to cxcl11 (Zlotnik and Yoshie, 2000). Studies of a cxcR3 null mouse showed the importance of the cxcR3 pathway in wound healing in vivo (Hancock et al., 2000). In fact, full thickness wounds made in this mutant seem to fail to re-form its epithelium properly due to the formation of a hypercellular epidermis, supporting the hypothesis that the cxcR3 pathway induces a signal to stop migration in order to start cell differentiation (Yates et al., 2009; Yates et al., 2007).
6.2 Homeostatic chemokines during embryogenesis

Interestingly, none of the mammalian chemokines except cxcl12 and cxcl14 possess clear orthologues in any other vertebrate class (Bleul et al., 1996; Campbell et al., 1998). cxCR4 is mostly involved in cell migration during embryonic development. Of all CXC-ligands, cxcl12 is the most widely expressed in embryonic tissues, including the whisker pad mesenchyme (Belmadani et al., 2009), vessels, lungs, developing heart, and the developing central nervous system (McGrath et al., 1999). These findings explain the phenotype of mice lacking cxCR4 or cxcl12 where numerous structures in the central nervous system as well as in the heart and large vasculature fail to develop normally owing to deficits in progenitor cell migration, hence provoking the death of the embryo in utero (Ma et al., 1998; Zou et al., 1998). In zebrafish, the cxcl12/cxCR4 axis induces the neuromast deposition along the posterior lateral line (David et al., 2002). In this system, migration depends on the interaction between the chemokine cxcl12, which labels the path of migration, and its receptor cxCR4, which is present on the migrating cells (Dambly-Chaudiere et al., 2007; David et al., 2002). cxcl12/cxCR4 interactions also underlie other long-range migration events such as the movement of germ cells both in fish (Knaut et al., 2003) and in mouse (Molyneaux et al., 2003) and the migration of facial motoneurons in fish (Sapede et al., 2005). cxcl14, still an orphan ligand, is expressed at multiple sites in the embryonic ectoderm, including hair and vibrissae (Banisadr et al., 2011; Garcia-Andres and Torres, 2010); however, the cxcl14-/- mouse does not seem to have any developmental abnormalities (Meuter et al., 2007; Nara et al., 2007).
Aims of the study

The aim of this thesis was to study novel EdaA1 pathway target genes in order to better understand the gene interplays leading to the formation of hair. The specific aims were:

- To validate microarray results and analyze the specific expression patterns of newly found targets.
- To understand the function and role of those new targets during hair development.
- To identify other possible TNF pathways (apart from Eda and Troy pathways) involved in hair follicle formation.
Materials and methods

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<td>bp 49 to 695</td>
<td>III</td>
</tr>
<tr>
<td>cxcl11</td>
<td>NM_019494.1</td>
<td>bp 29 to 705</td>
<td>III</td>
</tr>
<tr>
<td>cxcR1</td>
<td>NM_178241.4</td>
<td>bp 468 to 1085</td>
<td>III</td>
</tr>
<tr>
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<td>NM_009909.3</td>
<td>bp 1140 to 2006</td>
<td>III</td>
</tr>
<tr>
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<td>NM_009910.2</td>
<td>bp 612 to 1179</td>
<td>III</td>
</tr>
<tr>
<td>cxcR4</td>
<td>NM_009911</td>
<td>bp 11 to 557</td>
<td>III</td>
</tr>
<tr>
<td>cxcR5</td>
<td>NM_007551.2</td>
<td>bp 1033 to 1686</td>
<td>III</td>
</tr>
<tr>
<td>cxcR6</td>
<td>NM_030712.4</td>
<td>bp 194 to 1026</td>
<td>III</td>
</tr>
<tr>
<td>cxcR7</td>
<td>NM_007722.3</td>
<td>bp 626 to 1138</td>
<td>III</td>
</tr>
<tr>
<td>Dkk4</td>
<td>BC018400</td>
<td>bp 114 to 1107</td>
<td>I</td>
</tr>
<tr>
<td>Edar</td>
<td></td>
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</tr>
<tr>
<td>GITR</td>
<td>NM_009400.2</td>
<td>bp 113 to 975</td>
<td>This work</td>
</tr>
<tr>
<td></td>
<td>NM_021985.2</td>
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<td></td>
</tr>
<tr>
<td>Lrp4</td>
<td>NM_172668</td>
<td>bp 60 to 883</td>
<td>I</td>
</tr>
<tr>
<td>Shh</td>
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<td>Sox4</td>
<td>NM_009238</td>
<td>bp 1111 to 1468</td>
<td>III</td>
</tr>
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<td>bp 537 to 1087</td>
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</tr>
<tr>
<td>Wnt10b</td>
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<td></td>
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</tr>
</tbody>
</table>

Edar: Laurikka et al., 2001

GITR: Laurikka et al., 2001

Lrp4: Laurikka et al., 2001

Shh: Vaahtokari et al., 1996

Sox4: Vaahtokari et al., 1996

TnfR1a: Vaahtokari et al., 1996

Wnt10b: Vaahtokari et al., 1996
Results and discussion

1. **Eda induces genes in all major pathways involved during development (I, III)**

Description of the microarray

In 2004, our laboratory showed that adding the Fc-EdaA1 protein onto E13 Eda null skins allows rescuing of the Eda null phenotype and induces the formation of hair placodes one day after addition of the protein into the culture media (Mustonen et al., 2004). This experiment showed that the Fc-EdaA1 protein is sufficient to induce its own pathway and its downstream target genes in vitro. In 2007, Pummill et al. showed that it was possible to identify Eda target genes by adding Fc-EdaA1 on E14 skins for few hours using the hanging drop technique (Pummill et al., 2007). This technique takes advantage of the relatively big E14 back skin, which allows us to cut this organ into two halves and use one half as a control and the other half as a treated sample. This way it is possible to analyze genes differentially expressed after Fc-EdaA1 induction in the same embryo. Using this method, Pummill et al. showed that a known feedback inhibitor of the Eda/NFκB pathway is expressed at the site of Edar in the hair placode: IκBα (Schmidt-Ullrich et al., 2006) was rapidly upregulated upon incubation with EdaA1 protein already 1h after induction and its expression reached its maximum at 4h (Pummill et al., 2007).

In order to identify the direct target genes of Eda, we used this technique combined with a genome wide microarray screen of the mouse genome. We decided to use two time points: 90 min and 4h to detect the immediate effects of EdaA1 protein addition on gene expression. The Affymetrix® microarray chips used contained approximately 14,000 mouse probes. In our settings, only probes showing a >1.5-fold difference and p-value <0.05 were considered as putative transcriptional target genes. An upregulation in 23 probes representing 22 genes was observed after 90 minutes and in 168 probes representing 126 known genes after 4h, only 2 genes downregulated after 1.5h and 44 after 4h. Of the 22 genes upregulated after 90 min, 16 were upregulated also after a 4h Fc-EdaA1 treatment (see Table 1 for description of some of the genes). The genes upregulated by Fc-EdaA1 included IκBα, and TGFβ family antagonists CTGF and follistatin that had previously been shown to be induced by Eda by qRT-PCR (Mou et al., 2006; Pummill et al., 2007), indicating that the approach was successful.

**Comparison with other screens aiming at Eda target gene identification**

Other genome wide studies aiming at discovering Eda target genes have been performed, but showed very different gene lists. These differences may be attributed to the different techniques used to discover Eda target genes. In fact, other groups have either compared the expression of genes in the adult skin of mice with altered levels of Eda expression and compared it to WT (Cui et al., 2002) or more recently have analyzed differentially expressed genes at various developmental stages in WT vs. Eda null skin (Cui et al., 2006). These two studies also aimed at discovering genes differentially regulated by
the Eda pathway but comparison between adults or embryos is likely to reflect some compensation mechanisms for the loss of Eda, as well as some functions in the maintenance of skin appendages in adult mice (Cui et al., 2002).

Schlessinger’s laboratory found that members of the Shh signaling pathway were differentially expressed between Eda null and WT skin (Cui et al., 2006). Pummila et al., also showed that Eda induces a rapid response from Shh (Pummila et al., 2007). However, our microarray did not list Shh as a potential Eda target. In fact, Shh showed an upregulation greater than 1.5 in two of the three microarrays; however, the p-values associated with each fold of inductions were too high to pass through the filters applied to the microarray and Shh was excluded from our gene list.

Looking back at the microarray results, it seems that Ptc2 was upregulated after 4h in two of the three microarrays we performed. Ptc2 was not reaching the 1.5 fold induction threshold, thus, while combining all three folds, Ptc2 induction was too low to allow it to belong to the list. However, some common genes were identified by both approaches such as Ltbβ and Dkk4 (Cui et al., 2006). Dkk4 will be discussed in more detail in the next chapters.

**Overview of differentially expressed genes**

Differentially expressed probes represented genes in many signaling pathways: Wnt, fibroblast growth factor (Fgf), transforming growth factor β (TGFβ), TNF superfamily, and other families such as epidermal growth factor (Egf), chemokines, SRY-box containing genes (Sox), and cell adhesion molecules (CAMs) (Table 1).

Several TNF superfamily members were upregulated after 4h of induction by Eda (Table 1). Expression of Edar was upregulated as previously shown by qRT-PCR in embryonic skin explants (Mou et al., 2006). Eda pathway activation also seems to activate other TNF ligands such as TNFα and TNFRs such as Fas, Tweak, and GITR, but their involvement in ectodermal organ formation is yet to be addressed. As Eda signals mostly through NFκB, we expected to see the upregulation of different NFκB pathway components such as p50 and IκBα to prove the induction of the Eda pathway by the protein (Table 1) (Pummila et al., 2005). A20, a NFκB feedback loop inhibitor (Shembade et al., 2010), was also upregulated. qRT-PCR analysis showed rapid upregulation of A20 (Table 1), which together with the fact that A20 is a known target of NFκB pathway (Verstrepen et al., 2010), indicates that it is likely a direct transcriptional target of Eda. Furthermore, we also demonstrate that EdaA1 protein was able to significantly induce A20 protein levels in WT back skin epithelium as revealed by Western blot analysis.

Icam1 and MadCAM1, two cell adhesion molecules, were upregulated after 4h incubation with the protein Fc-EdaA1. Both genes were previously highlighted as potential Eda target genes (Nishioka et al., 2002). This experiment was based on the expression patterns of Icam1 and Madcam1 in the E14 mouse embryo. Both genes showed a patterned expression in the E14 embryo at the site of Edar expression. Interestingly, this expression was lost in both Eda null and Traf6 null mouse embryos. Furthermore, skin culture experiments allowed this group to postulate that both Icam1 and MadCAM1 expression occurs concomitantly with Eda signaling activation (Nishioka et al., 2002).
Icam1 and Madcam1 have NFκB binding sites in their promoter regions (Bunting et al., 2007; Takeuchi and Baichwal, 1995). TNFα is known to induce the expression of both molecules, most likely in an NFκB-dependant manner (Takeuchi and Baichwal, 1995). Therefore, it is likely that both Icam1 and MadCAM1 are direct target genes of the Eda pathway. However, their role during hair placode development still needs to be addressed.

Wnt pathway
According to the microarray, Eda seemed to induce two Wnt inhibitors: Dkk4 and Lrp4. These results were somewhat surprising as Eda and Wnt pathways were always thought to work in parallel in order to induce placode formation and patterning during ectodermal organ development (Narhi et al., 2008). However, our results confirmed the involvement of the Eda pathway in restricting Wnt signaling (for further discussion see below).

In addition, several members of the Wnt pathway were downregulated after 4h exposure to the protein EdaA1, including Wnt6, Fzd6, Rsps3, and Sostdc1. Sostdc1 downregulation was validated using qRT-PCR. Sostdc1 (ectodin or Wise), is a BMP and Wnt inhibitor expressed in the developing ectodermal organs but its expression is restricted to the surrounding of hair placodes (Itasaki et al., 2003; Laurikkala et al., 2002; Mou et al., 2006; Narhi et al., 2008). According to our results, Eda seems to participate in the exclusion of Sostdc1 from the placode.

Wnt6 has been shown to induce Eda expression in tooth mesenchyme and hair epithelium during early placode development (Laurikkala et al., 2002). The fact that Wnt6 could be inhibited by the Eda has, so far, not been addressed.

Wnt10a and Wnt10b were not listed in the microarray and further analysis of Wnt10b fold of induction using qRT-PCR showed a mild induction of 1.46 and 1.52 after 3h and 5h respectively; therefore, it was not considered as being an Eda target gene. However, using different time points (and maybe using another batch of Fc-EdaA1 protein), I showed that Wnt10a and Wnt10b were slightly upregulated after 2h induction by Eda and that this upregulation was lost after 4h. A statistical analysis was then performed and proved that the induction of Wnt10a and Wnt10b after 2h incubation with EdaA1 was low (1.4 and 2.1-fold respectively), but significant (Voutilainen et al., 2012).

FGF pathway
Two genes implicated in the FGF pathway were upregulated: Fgf20 and Dusp6. Interestingly, both genes have not yet been linked to ectodermal development. However, our laboratory has validated Fgf20 as a potential Eda target (Haara et al., submitted; Mikkola and Lindfors, unpublished). Study of an Fgf20 -/- mouse has revealed a tooth crown phenotype similar to the one seen in the Eda null mouse (Haara et al., submitted; Pispa et al., 1999). Fgf20 expression during molar embryonic development has been reported and it seems to be restricted to the primary and secondary enamel knots (Porntaveetus et al., 2011). However, the function of Fgf20 during tooth morphogenesis and hair development still needs to be understood.
Table 1. List of the selected genes differentially expressed in the microarray and their validation using qRT-PCR. The significance of gene induction was also tested using statistical analysis; all genes significantly upregulated are marked with an *, all others were not tested.

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Gene</th>
<th>Fold of increase (90min)</th>
<th>Fold of increase (4h)</th>
<th>Fold of increase (2h)</th>
<th>Fold of increase (4h)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Microarray</strong></td>
<td></td>
<td><strong>qRTPCR</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TNF/NF-kB pathway</strong></td>
<td>Ltβ</td>
<td>3.35</td>
<td>6.11</td>
<td>7.98*</td>
<td>21.05*</td>
</tr>
<tr>
<td></td>
<td>Tnfα</td>
<td>-</td>
<td>2.11</td>
<td>9.14*</td>
<td>9.19*</td>
</tr>
<tr>
<td></td>
<td>Edar</td>
<td>-</td>
<td>3.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fas</td>
<td>-</td>
<td>1.99</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fn14</td>
<td>-</td>
<td>1.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gitr</td>
<td>-</td>
<td>2.92</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NfκB; p50</td>
<td>-</td>
<td>1.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NfκB2; p52</td>
<td>-</td>
<td>1.58</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A20</td>
<td>-</td>
<td>1.74</td>
<td>3.64*</td>
<td>4.83*</td>
</tr>
<tr>
<td></td>
<td>IkBα</td>
<td>-</td>
<td>1.52</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NfκBβζ</td>
<td>-</td>
<td>1.54</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dkk4</td>
<td>-</td>
<td>30.41</td>
<td>12.73</td>
<td>45.66</td>
</tr>
<tr>
<td><strong>Wnt pathway</strong></td>
<td>Ectodin</td>
<td>-</td>
<td>0.57</td>
<td>0.95 (1h)</td>
<td>0.37 (5h)</td>
</tr>
<tr>
<td></td>
<td>Lrp4</td>
<td>-</td>
<td>1.87</td>
<td>1.12 (1h)</td>
<td>1.98 (5h)</td>
</tr>
<tr>
<td></td>
<td>CTGF; Ccn2</td>
<td>1.53</td>
<td>9.66</td>
<td>1.34 (1h)</td>
<td>3.36 (5h)</td>
</tr>
<tr>
<td><strong>TGFβ pathway</strong></td>
<td>CyR61</td>
<td>-</td>
<td>1.57</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fst</td>
<td>-</td>
<td>1.91</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>FGF pathway</strong></td>
<td>Dusp6</td>
<td>-</td>
<td>4.87</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fgf20</td>
<td>1.60</td>
<td>3.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ccl20</td>
<td>3.02</td>
<td>8.92</td>
<td>39.12</td>
<td>124.88</td>
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<tr>
<td></td>
<td>Cxcl1</td>
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<td>6.55</td>
<td>20.04</td>
<td>28.89</td>
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<tr>
<td></td>
<td>Cxcl2</td>
<td>1.72</td>
<td>4.33</td>
<td>15.62</td>
<td>13.68</td>
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<tr>
<td><strong>Chemokine pathway</strong></td>
<td>Cxcl9</td>
<td>-</td>
<td>6.26</td>
<td>2.98</td>
<td>3.86</td>
</tr>
<tr>
<td></td>
<td>Cxcl10</td>
<td>4.85</td>
<td>2.27</td>
<td>46.14</td>
<td>49.30</td>
</tr>
<tr>
<td></td>
<td>Cxcl11</td>
<td>6.90</td>
<td>5.92</td>
<td>17.76</td>
<td>46.68</td>
</tr>
<tr>
<td></td>
<td>Cxcl14</td>
<td>-</td>
<td>0.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Icam1</td>
<td>1.61</td>
<td>2.31</td>
<td>2.42*</td>
<td>4.89*</td>
</tr>
<tr>
<td></td>
<td>MadCAM1</td>
<td>-</td>
<td>2.17</td>
<td>1.43*</td>
<td>2.01*</td>
</tr>
<tr>
<td></td>
<td>Mmp9</td>
<td>-</td>
<td>4.70</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Other genes</strong></td>
<td>Pth-RP</td>
<td>-</td>
<td>7.45</td>
<td>8.2*</td>
<td>8.83*</td>
</tr>
<tr>
<td></td>
<td>Sox4</td>
<td>-</td>
<td>1.54</td>
<td>1.39*</td>
<td>1.39*</td>
</tr>
<tr>
<td></td>
<td>Sox9</td>
<td>-</td>
<td>2.03</td>
<td>1.25*</td>
<td>2.70*</td>
</tr>
<tr>
<td></td>
<td>Sox10</td>
<td>-</td>
<td>1.78</td>
<td>1.15 (NS)</td>
<td>0.81 (NS)</td>
</tr>
<tr>
<td></td>
<td>Sox21</td>
<td>-</td>
<td>4.59</td>
<td>1.08 (NS)</td>
<td>1.56*</td>
</tr>
<tr>
<td><strong>EGF</strong></td>
<td>Epgn</td>
<td>-</td>
<td>-</td>
<td>2.28*</td>
<td>3.77*</td>
</tr>
<tr>
<td></td>
<td>Areg</td>
<td>-</td>
<td>-</td>
<td>1.49*</td>
<td>2.03*</td>
</tr>
</tbody>
</table>
**EGF family**

Several EGF family members were highlighted as likely Eda targets in our microarray, notably, amphiregulin and epigen. Interestingly, EGF proteins were first qualified as hair placode inhibitors (Cohen and Elliott, 1963; Kashiwagi et al., 1997). In skin organ culture, administration of EGF ligands (EGF and TGFα) dramatically inhibits hair morphogenesis in E13.5 mouse skin (Kashiwagi et al., 1997). However, I have confirmed that Eda was able to induce a feeble but significant expression of amphiregulin and epigen in Eda null back skin. Their expression was barely detectable using whole mount in situ hybridization (Voutilainen et al., 2012). Further study of the effect of Areg and Epgn during mammary gland branching showed that Areg and Epgn increase the number of terminal end bud formation in E13 mammary gland explants cultured for 5 days with each protein. The importance of Areg and Epgn during branching could account for the mammary gland phenotype observed in the Tabby mouse, where the mammary tree shows fewer branches in the absence of Eda (Voutilainen et al., 2012). However, the involvement of Areg and Epgn during other ectodermal appendage development still needs to be understood.

**Sox family of transcription factors**

Several SRY-box containing gene (Sox) family members were upregulated; I confirmed Sox4, Sox9 and Sox21 upregulation using the qRT-PCR method and statistical analysis. A recent study highlighted a patterned expression of Sox4 in the developing hair placode at the site of Edar expression (Hoser et al., 2008). Our study confirmed this finding and also showed that Sox4 expression was lost from the Eda null mouse skin. However, Sox4 seems to also have a role in hair cycling, as its expression was detected in the bulge region of hair follicle in anagen phase (Lowry et al., 2005).

Sox9 was shown previously to be expressed during hair placode formation (Vidal et al., 2005) and our study points to its lack of expression in Eda null mouse skin. Interestingly, Sox9 has been shown to be one of the earlier markers of hair follicle stem cells in mouse, as Sox9 positive cells constitute the stem cell niche of the hair follicle in the bulge region (Vidal et al., 2005; Nowak et al., 2008). An interesting link between Tcf/β-catenin and Sox9 expression was made in the intestines where the absence of Sox9 prevents paneth cell differentiation (Bastide et al., 2007; Mori-Akiyama et al., 2007). Hence, Sox9 induction may have an importance in regulating the Wnt pathway during hair development and Eda induction of Sox9 could trigger the commitment of placodal cells to hair follicle formation fate.

The importance of Sox21 has mostly been studied postnatally as Sox21 null mice show progressive alopecia due to improper keratinization of the hair shaft cuticle that anchors the hair shaft, leading to the early shedding of Sox21 null mouse hair (Kiso et al., 2009).

**Chemokines**

The microarray highlighted for the first time a link between the Eda pathway and chemokines. One of the most interesting results of the microarray was the important upregulation of cxcl10 and cxcl11 and two other chemokines: cxcl1, cxcl2 already 90
min after induction with the protein Fc-EdaA1. cxcl11, cxcl2, and cxcl1 continued to be upregulated after 4h. cxcl9 and ccl20 were only found in the 4h list of genes upregulated upon EdaA1 induction. The importance of this discovery will be discussed in chapter 4.

2. **A20 is involved in the negative feedback loop of EdaA1 signaling (II)**

A20 (Tnfaip3) is an important modulator of Tnfa signaling. A20 is induced by NFκB after Tnfa activation and seems to be involved in the negative feedback loop of the NFκB pathway in order to stop TNFα signaling (Pasparakis et al., 2006; Shembade et al., 2010). As A20 was also highlighted in the microarray and confirmed as a potential Eda target using the qRT-PCR method, we studied its expression during embryonic development.

**A20 is expressed in the hair placode and suppresses Edar-induced NFκB signaling**

In the hair placode, A20 was expressed in the epithelium of the forming placode where Edar expression and NFκB activation are also visualized. Interestingly, its expression was not detectable in the mammary gland or tooth placodes, which may account for a lack of expression as well as very low levels of mRNA expression that could not be detectable using whole mount DIG in situ hybridization (WMISH).

Using an NF-κB-dependent luciferase reporter assay, we showed that A20 inhibits Eda-induced NFκB signaling, hence reinforcing the idea that A20 may act as a feedback inhibitor of the Eda pathway involved in terminating/restricting Eda signaling in hair placodes. Interestingly, the ability of A20 to suppress Eda signaling does not seem to depend on the N-terminal deubiquitination domain of A20, but requires its C-terminal domain. Likewise, a recent study showed that the C-terminal domain encompassing the E3 ligase domain is able to suppress NFκB activity (Bosanac et al., 2010).

**A20{EKO} mouse phenocopies partially K14-EdaA1 mouse**

Study of the A20{EKO} mouse phenotype, an epidermis-specific A20-deficient mouse showed that, despite the lack of an NFκB negative feedback loop, no sign of inflammation was detected in A20{EKO} mice. In fact, the NFκB pathway is important to control skin homeostasis and overactivation of this pathway gives rise to skin inflammation (Lippens et al., 2009; Pasparakis et al., 2006). On the other hand, histological analysis of the back skin revealed an increased proliferation and very low levels of apoptosis of the basal cells of the epidermis, consistent with overactivation of NFκB, which promotes cell survival under TNFα activation. The lack of inflammation or infiltrating immune cells showed that the absence of A20 could be compensated by other deubiquitinase enzymes, such as CYLD, to prevent spontaneous skin inflammation (Massoumi et al., 2006).

The most interesting phenotype of this mouse resided in the abnormal development of several ectodermal organs such as hair, Meiboian glands, and nails. Unlike their control littermate A20{fl/fl}, A20{EKO} showed disheveled hair, but still no sign of the hair
Results and discussion

loss or alopecia one would expect in case of hair autoimmune disease. A closer look at the hair type showed that contrary to normal mice, which have four kinds of hair (guard, awl auchene and zigzag), the A20EKO mouse had mostly one type of curly hair. Moreover, hair plucked from A20EKO fur was more fragile than from the control mutant and showed extended cuticle damage, detected by electron microscopy analysis. A similar hair phenotype was seen in K14-Eda and K14-Edar mice, along with the defects in Meiboian glands and thicker nails also seen in the A20EKO mouse (Mustonen et al., 2003). However, both the K14-Eda and K14-Edar mutants also show the development of extra teeth and nipples, but those features were never observed in A20EKO.

The similarities between the A20EKO phenotype and K14-Eda together with the identification of A20 as a feedback inhibitor of Eda pathway suggest that the A20EKO phenotype could be the consequence of over stimulated Eda signaling. The fact that A20 expression was never found in the mammary glands or teeth using in situ hybridization could explain the lack of tooth and mammary gland phenotype in the A20EKO mutant. Another explanation could be that the Cre line used to delete the A20 gene is not effective before E15 and it is known that the tooth and mammary gland placodes are starting to develop around E12; therefore, A20 deletion is too late to influence the development of those organs.

3. Interplay between Eda and Wnt pathways to regulate the expression of placodal genes: Dkk4 and Lrp4 (I)

Dkk4 was highly upregulated after 1h induction while Lrp4 was modestly induced by EdaA1 protein according to the microarray (Table 1). WMISH showed that both of these genes were co-expressed with Edar in skin appendage placodes, making the hypothesis of regulation by the Eda pathway more likely (Pispa et al., 2003; Pummila et al., 2007). Recently, several other groups also reported similar expression patterns for Dkk4 and Lrp4 (Bazzi et al., 2007; Sick et al., 2006; Weatherbee et al., 2006; Zhang et al., 2009).

In order to address if Eda can induce Dkk4 and Lrp4 directly, we screened for NFκB responsive elements in both Dkk4 and Lrp4 promoter regions by computational analysis to find evolutionary conserved sites between mice and humans (Sandelin et al., 2004). Three NFκB responsive elements (RE) were found in the promoter region of Dkk4 and six in the promoter region of Lrp4. To test whether Dkk4 could be a direct target of Eda, we cloned the 800bp promoter region containing all three NFκB RE into a promoter-less luciferase reporter vector and performed a promoter-reporter assay to test if the Eda pathway was able to induce Dkk4 expression. We could detect about 2-fold increase in luciferase expression when the Dkk4 promoter was co-transfected with Edar vector, whereas transfection with a vector expressing a truncated form of Edar (Edar sleek) compromised but did not abolish NFκB response (Koppinen et al., 2001) and gave rise to no clear induction of Dkk4 compared to the empty vector. This low responsiveness of the proximal Dkk4 promoter was surprising considering that, in skin explants, Fc-EdaA1 treatment had a more potent effect on Dkk4 transcription and suggested that additional NFκB binding sites may be present elsewhere. In line with this, a more recent study showed that an NFκB RE highly conserved between mouse and rat was encountered 2kb upstream of the Dkk4 transcription starting site (Zhang et al., 2009). Using chromatin
immunoprecipitation, Zhang et al., 2009 could demonstrate that NFκB binds directly to Dkk4 using this highly conserved RE. We can then postulate that if our construct would have been longer, the induction by NFκB we measured using the transfection assay would have been greater.

We then analyzed the expression of Dkk4 and Lrp4 in skin appendage placodes of both Eda null and K14-Eda embryos. Expression of both genes was more intense in K14-Eda hair placodes compared to WT. One of the most surprising findings concerning Lrp4 and Dkk4 embryonic expression was that, despite evidence that at least Dkk4 is a direct target of Eda, both Lrp4 and Dkk4 expression were still seen in Eda null embryos. Most importantly, these two genes were the first localized markers visible in Eda null embryos at this stage (Headon and Overbeek 1999; Laurikka et al., 2002) and were the first markers of pre-placodes that were previously identified by histology (Schmidt-Ullrich et al., 2006). However the patterning was different from the one seen in WT hair placodes. The expression patterns of both genes were fainter and although their expression was localized to specific areas, the circular shape of the expression pattern characteristic of a placode was lost in Eda null embryos. Analysis of NFκB reporter activity in Eda null embryos at E13.5, i.e., when hair placodes are just starting to emerge on both side of the mammary line, suggested a complete absence of NFκB signaling in the absence of Eda. However, no difference in Dkk4 and Lrp4 expression was observed between Eda null and WT embryos. Taken together our data suggested that the expression of Dkk4 and Lrp4 is also regulated by other pathways. These data also demonstrate that Eda is dispensable for early hair placode induction, which is thought to take place in response to the dermal signal (Mikkola and Millar, 2006) and reinforce the idea that the Wnt/β-catenin pathway is the earliest signal activated at the onset of hair placode formation (Zhang et al., 2009; Andl et al., 2002; Narhi et al., 2008).

Based on our transfection assay and the literature, we postulated that expression of Dkk4 and Lrp4 in Eda null embryonic skin was most certainly due to Wnt signaling induction of its own inhibitor (Sick et al., 2006; Bazzi et al., 2007). To test this hypothesis, we treated Eda null skin explants with BIO, a Wnt pathway activator (which inhibits GSK-3β), with or without Fc-EdaA1 protein and analyzed Dkk4 expression by qRT-PCR. In this setting, Dkk4 expression was induced via Wnt pathway alone and we observed an additive effect with Fc-EdaA1 further suggesting that both pathways can induce Dkk4 in mouse skin.

4. Do chemokines have a role in hair development? (III)

cxcl10 and cxcl11 are novel targets of the Eda pathway

All six chemokines highlighted by the microarray were confirmed using qRT-PCR. Out of those six chemokines only cxcl10 and cxcl11 showed a patterned expression in the epithelium of the developing placode. Interestingly and although Edar expression is also found in other ectodermal organs placodes such as the mammary gland and tooth, cxcl10 and cxcl11 expression could not be detected in these organs, thus showing that cxcl10 and cxcl11 were likely to be involved in hair development only.

WMISH using cxcl10 and cxcl11 RNA probes showed that their expression was undetectable in the Eda null mouse mutant and more intensely in the K14-Eda mouse
than in WT at E14. One may object that the lack of hair placode formation in the former and the increase in size of hair placode in the latter may account for the difference of expression. Therefore, we also analyzed the promoter region of cxcl10 and cxcl11 to test if both genes had an NFκB responsive element that could account for Eda activation.

Screening of the promoter region of both cxcl10 and cxcl11 showed several NFκB REs conserved between mouse and human, as well as mouse and rat. In this experiment, we showed that the Eda pathway induces 16-times and 21-times more expression of cxcl10 and cxcl11, respectively, compared to an empty vector, whereas a mutant form of Eda (Edamleek) induced only weakly both promoter regions. These data strongly suggest that the Eda pathway is able to induce cxcl10 and cxcl11 expression directly via NFκB signaling.

**Primary hair follicle patterning is affected in mice lacking CxcR3, the receptor for cxcl10 and cxcl11**

cxcl10 and cxcl11 share the same receptor, cxcR3 (Zlotnik and Yoshie, 2000). In this study we showed that the cxcR3 expression pattern correlates with cxcl10 and cxcl11 expression in the developing hair placodes and, just like its ligands, the cxcR3 expression pattern is undetectable in Eda null embryos using WMISH. Therefore, we studied the embryonic and adult phenotypes of the cxcR3 null mouse (Jackson laboratories cxcr3tm1Dgen/J mice, stock #005796) to analyze the effect of the lack of cxcl10 and cxcl11 signaling during hair development. According to migration studies of cells harvested from this mouse mutant, cxcR3 null cells are not able to respond anymore to cxcl10 and cxcl11 chemotaxis (Sorensen et al., 2010).

Analysis of the embryonic hair placode and adult hair shaft distributions allowed us to conclude that the lack of cxcR3 signaling during hair follicle development gives rise to sparser hair placodes at E14, as the number of hair placodes per surface area diminished by about 20%. In accordance with the embryonic phenotype, a decrease in the proportion of guard hair shafts from 3.8% in the littermate control to 2.2% in the cxcR3 null adult mouse was observed. Interestingly, the lack of the cxcR3 pathway only influenced the first wave of hair placode formation at E14. This could be explained by the fact that the number of transcripts of cxcl10 and cxcl11 in the skin dramatically decreases at the onset of subsequent waves of hair follicle formation at E16 and around birth. In addition, their expression in the second and third wave of hair follicle formation was not detectable using radioactive or DIG in situ hybridization on skin sections.

The mechanism by which CXC-chemokines regulate hair development is yet to be understood. Several studies on wound healing pointed to the cxcl10-cxcl11-cxcR3 trio as regulators of wound healing (Satish et al., 2003) and in vivo studies of cxcR3 deficient mice showed that lack of this specific trio impairs reepitheliazation of full thickness wounds (Yates et al., 2007; 2008) due to a lack of keratinocyte migration inhibition. In fact, it seems that the cxcR3 pathway is needed to slow down migration and induce differentiation of the cells invading the wound. Other cell studies conducted on epithelial mammary gland cancer cells pointed to cxcR3 pathway as a major factor responsible for epithelial cell invasion into the mesenchyme (Shin et al., 2010). Both mechanisms could be important for hair placode development but which one is yet to be discovered.
**Other cxcRs are not expressed in hair placodes**

The mild phenotype observed in cxcR3 null mouse prompted us to wonder if other cxcRs could compensate for the loss of the cxcR3 pathway. However, none of the cxcRs were expressed in a patterned manner in the embryonic epithelium. Interestingly, though, cxcR4 was found in the mesenchymal condensate at E14 and in the dermal papilla of the vibrissae as previously shown (Belmadani et al., 2009). A role for cxcR4 in development has been established, albeit not in ectodermal placode formation (Chalasani et al., 2003; Chong et al., 2007). However, cxcR4 seems to regulate the migration of hair melanocytes progenitors in mouse (Belmadani et al., 2009).

Interestingly, expression of cxcl14, an orphan chemokine ligand, was recently reported in the whisker epithelium and hair follicle ORS (Garcia-Andres and Torres, 2010; Banisadr et al., 2011). Although no hair placode expression was reported, it is tempting to speculate that its expression correlates with Edar in the hair placode. Hence, cxcl14 could be compensating for the loss of cxcl10/cxcl11 signaling in the cxcR3 null mouse. A cxcl14 null mouse has been generated, but no obvious developmental abnormalities have been reported (Meuter et al., 2007; Nara et al., 2007).

It is noteworthy that cxcl14 was also present in our microarray: it was one of the downregulated genes after 4h induction by Eda protein (Table 1). This downregulation was not confirmed using qRT-PCR, but it is interesting to speculate that, whereas Eda induces the expression of cxcR3 pathway in the placode, it downregulates cxcl14, which may also be expressed in the placode. It could be interesting to test the expression pattern of the cxcl14 gene in the developing embryo and most importantly in K14-Eda to see if its expression is decreased in this mutant.

**5. Eda has a role in hair placode patterning (I, II, III)**

The microarray profiling of Eda targets remarkably deepened our knowledge about the intricate pathways of genes involved in hair development. Interestingly, the Eda pathway, which promotes hair placode development at E14, seems to do so by inducing not only activators of hair placodes such as CTGF and follistatin (Bmp antagonists), cxcl10 and cxcl11, but also inhibitors such as Dkk4.

Our laboratory has shown earlier that addition of Eda protein in skin culture increased the size and distribution of hair placodes in a dose-dependent manner. Furthermore, study of K14-EdaA1 transgenic mouse showed that the first wave of hair placode is disturbed and hair placodes are larger and sometimes fused (Mustonen et al., 2003; Mustonen et al., 2004). This shows that Eda regulation is important for the first wave of hair placode patterning. According to the reaction diffusion model, the activator must induce its own inhibitor and itself and here we show that Eda induce A20 in order to inhibit NFκB signaling. However, the activator and inhibitor must be diffusible molecules and Edar signaling only affects the cell in which it is expressed. Our study and others showed that Eda signaling is able to induce soluble molecules that will regulate the patterning such as Dkk4 (described here; Mou et al., 2006), follstatin, and CTGF (Pummila et al., 2007; Mou et al., 2006) by acting on Wnt and Bmp pathways, respectively (Figure 10).
Results and discussion

Figure 10. Schematic representation of the possible interplay between Wnt, Eda and Bmp signaling and Eda target genes in order to fine tune hair placode patterning. At the onset of hair placode formation, the Wnt signaling pathway is induced in the pre-placode. Consequently, it induces the expression of its inhibitors, Dkk4 in the epithelium and Dkk1 in the mesenchyme (a). Wnt signaling also induces the expression of Edar at the future site of the placode around E14. In turn, Edar pathway triggers the activation of NFκB transcription factor and the expression of its target genes which induces more expression of Dkk4 in the epithelium in order to fine tune Wnt signaling to the placode site (b). Edar pathway activation in the placode also induces the expression of cxcl10 and cxcl11 which seem to act as placode formation activators. The Edar pathway also induces the expression of two Bmp inhibitors, CTGF and follistatin, and other Bmps in the mesenchyme. CTGF and follistatin expression excludes Bmp signaling from the developing placode while Bmp signaling in the mesenchyme triggers the condensation of mesenchymal cells. Bmp expressed in the surrounding of the placode, inhibit Edar expression thus confining Edar signaling to the placode and providing the correct patterning of the first wave of hair formation. Edar signaling also induces the expression of A20 and IκBα, two NFκB inhibitor, in order to regulate its own signaling in the developing placode (c). The events described in b) and c) are concommitent and were described separately for clarity purposes. Interactions marked with an asterix were described in this thesis.
The Eda pathway induces the expression of Bmp inhibitors. Interestingly, Bmp inhibition by Noggin is able to rescue, at least in part, primary hair placode formation in *Eda* null skin explants (Mou *et al.*, 2006; Pummila *et al.*, 2007). Furthermore, deletion of Bmp receptor in the epithelium increases hair follicle density in mouse (Andl *et al.*, 2004). Mou *et al.* suggested that interplay with Edar, Bmps, and CTGF regulates the patterning of hair follicles. It was proposed that Edar induces rapid induction of Bmp in the mesenchyme and CTGF in the epithelium. Bmp in turn downregulates Edar in the hair placode surroundings because Edar upregulates Bmp inhibitors in the nascent hair placode (Figure 10). All these interactions will then generate the primary hair follicle pattern (Mou *et al.*, 2006).

One interesting result is that Dkk4 is expressed in the pre-placode before Edar is localized. The interplay of Wnt/Eda signaling has been studied for its importance in placode development by us and others and one emerging scheme is that Wnt/β-catenin is important in inducing the follicular keratinocyte fate. Hence, when Wnt/β-catenin signaling is stabilized in the epithelium, it will give rise to ubiquitous hair placode induction (Narhi *et al.*, 2008; Zhang *et al.*, 2008). Zhang *et al.* (2009) also found that Edar expression was induced by the Wnt/β-catenin pathway. Hence, it seems that during hair follicle initiation, there is initially Wnt activation in the preplacodes (Schmidt-Ullrich, 2006; Mou *et al.*, 2006). Wnt then induces Edar expression at this site (Zhang *et al.*, 2009), but activation of NFκB by the Eda pathway will in turn activate placode activators (Wnt10a/b) and inhibitors of the Wnt/β-catenin pathway in order to fine tune the pattern (Mou *et al.*, 2006; Zhang *et al.*, 2009; Voutilainen *et al.*, 2012) (Figure 10).

Wnt also induces its own inhibitor, Dkk4 in the epithelium and likely Dkk1 in the mesenchyme, and studies have shown that overexpression of moderate levels of a Dkk protein (Dkk1 or Dkk2) in the epithelium gave rise to sparser placodes during the first wave of hair placode induction, whereas high levels completely block hair follicle induction (Andl *et al.*, 2002; Sick *et al.*, 2006). We showed in skin explant cultures that high amounts of Dkk4 protein inhibited primary hair placode formation. Interestingly, Wnt and Eda share the same target genes Dkk4 and Lrp4, as both of them show NFκB and Lef1 responsive elements in their promoter regions. Taken together, these three pathways, Wnt, Bmp, Eda, seem to play an important role in patterning and seem to be interlinked in multiple ways via cross-regulation.

We also showed an increase in hair placode spacing in mice lacking *cxcR3*, suggesting that *cxc10* and *cxc11* function as hair placode activators (Figure 10). Our findings imply, in correlation with Turing’s model (Turing 1952), that the lack of *cxc10* and *cxc11* during this reaction-diffusion event imbalances the threshold of the activators. Therefore, it will impair the initial phase of oscillation that finally will modify hair placode patterning. Nevertheless, the mild phenotype of the *cxcR3* null mouse could be explained by the fact that Eda is inducing many other pathways also responsible for hair development that could compensate to a certain extent for the loss of the *cxcR3* pathway. Crossing of the *cxcR3* null mouse line with K14-*Eda* did not have any effect, possibly due to the overexpression of many other pathways, which are compensating for the loss of one class of activators.

Therefore, it seems that many pathways are intricately regulating the pattern of hair follicle formation. Wnt, Bmp, and Eda seem to be the most important pathways in this
regulation, but they also seem to interplay with one another, rendering mathematical modeling of hair placode patterning challenging (Klika et al., 2012). However, increasing the knowledge about all pathways involved and their particular spatial and temporal interactions will help us to understand how patterning is regulated and improve the the modeling of this process.

6. Identification of other TNFR pathways involved in the third wave of hair follicle induction

Studies of two different mouse mutants, the Eda/troy double knockout (Pispa et al., 2008) on one hand, and IkBaΔN mutants (Schmidt-Ullrich et al., 2001) on the other hand, have suggested that in addition to Edar and Troy, other TNF receptors could regulate hair placode formation. The Eda/troy double mutant does not show any sign of hair placode formation until E18; hence, both primary and secondary hair placodes fail to form (Pispa et al., 2008). In IkBaΔN mutants, the primary hair follicles do not form, reflecting the necessity of Edar-induced NFκB signaling in these follicles. Interestingly, the secondary hair follicles do develop, but the number of hair placentces of the third wave is severely reduced in those mutants (Schmidt-Ullrich et al., 2001). Therefore, we hypothesize that the NFκB pathway is also involved in the third wave of hair follicle development and that additional TNFR could function redundantly with Edar and/or Troy.

Selection of the receptors

The TNF superfamily comprises 29 receptors in humans and 27 in mouse (Figure 6) (Bossen et al., 2006). The expression of Edar, Xedar, and Troy in hair development has been extensively studied (Laurikkala et al., 2002; Pispa et al., 2008). Receptors missing a transmembrane domain which do not induce any pathway, were also discarded from the receptor list. In order to select the most interesting candidates, we decided to analyze the expression of each receptor by RT-PCR in E18 embryonic skin, at the time when tertiary hair placentces are induced. In addition, I verified the quality of primers using several adult tissues where the gene was known or suspected to be expressed. If the first set of primers was not amplifying the right size of PCR product, another set of primers was designed and ordered. All selected region from the 22 receptors were amplified from E18 skin (data not shown) except TnfRh3, which was never amplified no matter what tissue was used. OX-40 was successfully amplified from adult organs but not from embryonic skin. Therefore, those two genes were excluded as candidate genes.

As one may expect, the TNFR involved in E18 hair placode induction should be expressed in the skin epithelium, just like Edar and Troy. Therefore, I next separated the E18 epithelium from the mesenchyme and compared the TNFR number of transcripts from the epithelium with the whole back skin in order to see which of the receptors would be enriched in the epithelium at this stage.

So far, I have analyzed 12 receptors using this method and, among those six genes: TNFRI, CD27, GITR, DR5, BCMA and HVEM (Light-R) were enriched in the epithelium at E18 (Figure 11). Interestingly, GITR was found to correspond to the microarray list of genes induced by Eda after 4h incubation (Table 1).
Radioactive in situ analysis of semi finalists

The expression of four genes (TNFRI, BCMA, CD27 and GITR) so far was analyzed using radioactive in situ on skin sections of E18 and E19 wild type and E18 Eda/Troy double mutant that only showed the third wave of hair follicle development (Figure 12). TNFRI, CD27 and BCMA expression was weakly seen throughout the epithelium of both mutant and wild type mice. However, GITR expression was restricted to the hair follicle and interestingly was also seen in the epithelium of newly formed placodes in WT, as well as in Eda/Troy double mutant skin (Figure 12). Previous study has shown a similar expression of GITR protein in newborn skin and at E15 in the epithelium of the hair placode (Wang et al., 2005). However, GITR−/− mice show no defects in hair follicle formation and no accentuation of the Edar null phenotype was observed in compound Edar/GITR mutants (data not shown in Wang et al., 2005). However, because Troy expression is known to be localized to the third wave of hair placode, as well, compensation by Troy cannot be ruled out.

Therefore, further studies are needed to confirm or disprove the importance of GITR during hair follicle development. One possibility will be to inhibit GITR in skin explant culture at E17 before the third wave of hair follicle formation and see if the induction of the third wave is compromised. One possible way to address this question is to use...
the GITR ectodomain (Schneider et al., 2003) to compete with GITR signaling on WT or Eda/Troy double mutant developing back skin. Another possibility is to generate the Edar/Troy/GITR triple knockout in order to analyze the hair phenotype.

**Figure 12.** Expression pattern of TNFRI (a), BCMA (b), CD27 (c) and GITR in Wild type (WT) E18 and E19 back skin and in Eda/Troy double knock out E18 back skin. The placode or hair germ is indicated (arrow) and a close up of the placode or germ is shown (insert). Scale bar 100μm.
Conclusions and future directions

1/ The Eda pathway regulates expression of genes in all major pathways involved in hair development, including Wnt, FGF, and TGFβ/Bmp (I, III).

2/ Eda is a key player fine tuning hair placode formation and may exert its function by regulating the expression of both activators (cxcl10, cxcl11, CTGF) and inhibitors (Dkk4) of placode formation (I, III).

3/ Eda inhibits the Wnt pathway in order to limit its signaling to the placodal area during the first wave of hair follicle formation. Wnt seems to play a role during hair placode initiation and Eda is important for the fine tuning of hair placode patterning at E14 (I).

4/ Eda induces the expression of CXC-chemokines cxcl10 and cxcl11 and lack of signaling by the two chemokines leads to sparser hair placode distribution (III).

5/ Eda induces its own inhibitor A20 during hair development, most likely to terminate the Eda signaling pathway by inhibiting NFkB stabilization. The role of A20 downstream of Eda surprisingly seems to mostly be limited to hair development (II).

6/ Eda and Troy have already been shown to have a role in the first and second wave of hair formation, but what about other TNFRs? In this study I show that GITR is expressed during the third wave of hair placode formation (UD).

Taken together, the results presented in this study deepen our knowledge of the Eda pathway and its role during hair development and patterning. The Wnt pathway is known to be very important both for the induction and patterning of hair follicles. In this study, I propose Eda is a key regulator of hair placode patterning. I showed that Eda is able to induce its own inhibitor. However, whether the lack of Eda inhibitor A20 influences patterning, per se, is not known. It would be of interest to study hair placode formation in A20EKO mutants.

Eda also controls other pathways in order to restrict their signaling spatially and temporally. Eda restricts Wnt expression to the placodal area by inducing Wnt inhibitors Dkk4. This is the mechanism to exclude Wnt expression from the interplacodal area and ensure normal patterning. Increasing our knowledge about differential gene regulation and interaction will give us a better understanding on pattern formation and will help improving the Turing model. Other genes in the microarray are currently being studied in our laboratory and will most likely shed some more light on the complex interplay of major pathways in hair formation and patterning of hair placodes.

This study also finally puts Eda more firmly into the TNF superfamily. In fact, Eda has always been treated as an outsider because it does not induce any inflammatory response or apoptosis. Here, we show for the first time that it induces the expression of
chemokines like TNFα, the founding member of TNFs. Further studies of the role of those chemokines during development need to be addressed and it would be interesting to analyze the localization of γδ-Tcells (that express cxcR3) during hair development in order to see if these cells participate in placode formation. cxcl10 and cxcl11 could direct the migration of cells but this also needs further study. To this end, it would be interesting to analyze cell migration in tissue culture using live cell imaging and beads soaked with either or both cxcl10 and cxcl11.

This thesis was also a quest to find additional TNFRs that could be involved in the third wave of hair formation. This led us to discover that GITR was expressed in the epithelium of newly formed hair placodes. However, this project is still ongoing and we still need to analyze more detail the expression of other TNFRs. The expression of GITR and other TNFRs (if we find any) in mouse will be analyzed, as well as some functional analyses in order to validate GITR and/or others as inducers of the third wave of hair formation.
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