Ectodysplasin in Epithelial Morphogenesis: from Tabby to TNFs

Johanna Pispa

Developmental Biology Programme,
Institute of Biotechnology
and
Department of Biosciences,
Division of Biochemistry,
University of Helsinki

Academic Dissertation
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Helsinki 2004
For my mother, in memoriam
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ABBREVIATIONS

aa = amino acid
ADULT = acro-dermato-ungual-lacrimal-tooth syndrome
AEC = ankyloblepharon-ectodermal defects-cleft lip/palate
AER = apical ectodermal ridge
AP-1 = activator protein-1
βgal = beta-galactosidase
BMP = bone morphogenetic protein
cDNA = complementary DNA
cr = crinkled
DD = death domain
dl = downless
E = embryonic day
ED = ectodermal dysplasia
EDA = ectodermal dysplasia, anhidrotic
EEC3 = ectrodactyly, ectodermal dysplasia, and cleft/lip palate syndrome 3
FGF = fibroblast growth factor
HED = hypohidrotic ectodermal dysplasia
HED-ID = HED with immunodeficiency
Hh = hedgehog
IκB = inhibitor of NF-κB
IKK = IκB kinase
JNK = Jun N-terminal kinase
K14 = keratin 14
LEF1 = lymphoid enhancer factor 1
LMS = limb-mammary syndrome
LT-α,β = lymphotoxin-α,β
MAP = mitogen-activated protein
mRNA = messenger RNA
Msx = vertebrate homologue of Drosophila mucsle segment (Msh) gene
NIK = NF-κB inducing kinase
NF-κB = nuclear factor κB
OL-EDA-ID = ectodermal dysplasia, anhidrotic, with immunodeficiency, osteopetrosis, and lymphedema
OMIM = Online Mendelian Inheritance in Man
OPG = osteoprotegerin
PCR = polymerase chain reaction
RANK = receptor activator of nuclear factor κB
RANKL = receptor activator of nuclear factor κB ligand
Shh = sonic hedgehog
Ta = Tabby
TNF = tumor necrosis factor
TRAF = TNF receptor associated factor
TRAIL = TNF-related apoptosis-inducing ligand
UTR = untranslated region
LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original articles, which are referred to in the text by their Roman numerals. In addition, some unpublished data are also presented.


* Equal contribution.
ABSTRACT

Ectodermal organs, such as teeth and hair, share common developmental mechanisms. They all begin organogenesis as two adjacent tissue layers, the epithelium and the mesenchyme. An epithelial thickening, a placode, is formed, which then will bud into or out of the mesenchyme. Morphogenesis continues as a co-ordinated process of epithelial folding and branching, mesenchymal condensation, and differentiation of both tissues. The development of these organs is regulated by interactions between the epithelium and the mesenchyme, and by the transient signalling centres that form in the epithelium. Several common signalling factors from the BMP, FGF, Hh, and Wnt families are required for their development. Studies performed in this thesis and by others have shown that members of the TNF signalling pathway also contribute to ectodermal organogenesis.

Ectodermal dysplasias are congenital diseases where at least two ectodermal appendages are defective. In this study the murine gene, Tabby, for one of these dysplasias, X-linked hypohidrotic ectodermal dysplasia (HED or EDA), was cloned and shown to be a member of the TNF superfamily. The protein product was named ectodysplasin. The ectodysplasin mRNA expression pattern was studied initially in teeth and hair, and also in other embryonic tissues comparing it to its receptor, Edar. Ectodysplasin was expressed in the epithelium of the developing tooth, hair, lacrimal gland, and brain. However, in the salivary gland the mRNA was found in the mesenchyme. Edar expression was always found in the epithelium. The expression patterns suggest that ectodysplasin-Edar signalling can occur both between epithelial compartments and between the epithelium and the mesenchyme.

Tabby, the mouse ectodysplasin mutant, has defective tooth formation, specifically the cusp number of the first molars is reduced. Analysis of the molar tooth phenotype with molecular markers showed a reduction in the size of the tooth signalling center, the enamel knot. This gives support to the hypothesis that the enamel knot controls the size of the tooth crown. The reduction in Tabby molar cusp number could be partially rescued in vitro by application of exogenous FGF.

The potential role of other TNF signalling molecules was explored by studying the mRNA expression pattern of a TNF receptor, TNFRSF19, related to Edar. TNFRSF19 was co-expressed with Edar in the tooth, mammary glands, whiskers, and the limb bud suggesting that the two receptors may be functionally redundant in these organs.

Overexpression of Edar in transgenic mice affected tooth and hair formation in a dose-dependent manner. The primary hair follicle formation was inhibited, possibly by the upregulation of BMP4 throughout the developing hair epithelium.

In summary, work in this thesis includes the cloning of a novel protein involved in ectodermal organogenesis, ectodysplasin, a TNF superfamily member. The expression pattern of ectodysplasin and other TNF signalling pathway members was studied with in situ hybridisation. In vitro and in vivo studies with mutant and transgenic mice tissues have contributed to knowledge about this developmentally new signalling mechanism.
1. REVIEW OF THE LITERATURE

1.1. Ectodermal organ development

Teeth and hair have little in common in appearance, but developmentally they resemble each other considerably. Together with whiskers, feathers, scales, and mammary, sweat, lacrimal and salivary glands they derive from the ectoderm, and are thus called ectodermal organs (for reviews, see Chuong 1998; Millar 2002; Thesleff and Mikkola 2002; Pispa and Thesleff 2003; Veltmaat et al. 2003). The early morphogenesis of all ectodermal organs begins from two adjacent tissue layers, the epithelium and the mesenchyme, and inductive interactions between these tissue layers direct development in a sequential manner (Figure 1).

![Figure 1. Development of ectodermal organs.](image)

Morphologically very different organs, such as tooth, hair, mammary gland, and feather, develop from two adjacent layers: the epithelium (E) and the mesenchyme (M). An epithelial placode is formed, which then buds either into or out of the mesenchyme. During morphogenesis the epithelium and the mesenchyme proliferate and branch determining the final shape of the organ.

In most organs, the first signals instructing organ formation derive from the mesenchyme. An ectodermal placode is then formed by a local thickening of the epithelium. The placode will direct underlying mesenchymal cells to condense around the placode and to form a so-called papilla. The placode grows into an ectodermal bud, which projects either into (e.g. hairs) or out of (feathers) the mesenchyme. Subsequent folding and growth of the epithelium and the mesenchyme will contribute to the final shape of the organ, accompanied by organ-specific differentiation. The placode can be seen as a signalling center as it specifically expresses several growth factors.

The last twenty years of developmental biology have shown that members of the four signal transduction families, bone morphogenetic protein (BMP), fibroblast growth factor (FGF), hedgehog (Hh), and Wnt families are repeatedly required for development. Ectodermal organs are no exception to this rule (Millar 2002; Thesleff and Mikkola 2002; Pispa and Thesleff...
It is interesting that the expression patterns of many of these signals are also conserved between different types of organs. For example, alligator scale placodes express $Shh$ and $Bmp2$ in a very similar manner as chicken and duck scales and feathers (Harris et al. 2002).

### 1.1.1. Tooth development

Mammalian teeth develop from the oral ectoderm and the underlying neural crest-derived mesenchyme in the fronto-nasal process and the first branchial arch (Thesleff and Mikkola 2002; Cobourne and Sharpe 2003). Teeth can only form within the limits of the dental lamina, a U-shaped epithelial ridge in both maxilla and mandible. Four different types of teeth exist: incisors, canines, premolars, and molars. In each jaw quarter humans have two incisors, one canine, two premolars and three molars, whereas mice have only one incisor and three molars. Between the murine incisors and molars is a toothless diastema region. In the maxillary diastema tooth development is initiated but it soon ceases, and the diastema tooth buds degenerate by apoptosis (Keränen et al. 1999).

Hox proteins are involved in patterning of the vertebrate anterior-posterior body axis. In the maxilla and mandible it seems that the distalless homeobox proteins (Dlx) are required for patterning instead of Hox proteins. $Dlx$ genes are expressed in overlapping domains throughout the first branchial arch. $Dlx1/Dlx2$ mutant mice do not develop maxillary molars, and $Dlx5/Dlx6$ mutant mandibles have maxillary identity (Thomas et al. 1997; Depew et al. 2002). The identity of molars versus incisors may also be determined by mesenchymally expressed transcription factors, as ectopic Barx1 expression seems to transform incisors into molars (Tucker et al. 1998). The molecules determining identity of canines and premolars in e.g. humans are not known.

Tooth development can be divided into the initiation, bud, cap, and bell stages, named after the appearance of the epithelial component (Figure 2). In mouse molar teeth the initiation stage begins at embryonic day 11 (E11) when the lamina thickens to form a placode at the site of the future first molar. Mesenchymal cells will then condense around the epithelial placode to form a papilla, which is accompanied by upregulation of cellular proliferation and expression of extracellular matrix molecules such as syndecan and tenascin-C (Vainio and Thesleff 1992; Sahlberg et al. 2001).

Tissue recombination experiments have shown that the first identifiable inductive signal comes from the epithelium in contrast to most other ectodermal organs (Mina and Kollar 1987; Lumsden 1988). However, transplantation of mouse pre-migratory neural crest cells into chick embryos can induce tooth-like structures in the normally toothless chick jaw (Mitsiadis et al. 2003), suggesting that early mesenchymal signals may also be required. The exact nature of the first epithelial signal is not known but at least the balance of FGF and BMP signalling seems to regulate the sites of tooth formation (Neubüser et al. 1997; Mandler and Neubüser 2001). Conditional inactivation of $Fgf8$ in the first branchial arch ectoderm or inhibition of FGF signalling in vitro by an FGFR1-specific inhibitor results in loss of tooth development (Trumpp et al. 1999; Mandler and Neubüser 2001). FGFs and BMPs regulate several targets in the dental mesenchyme or epithelium, some of them antagonistically. Some of these target transcription factors, e.g. Msx1/Msx2, Pax9 and Pitx2, are essential for tooth development (Vainio et al. 1993; Neubüser et al. 1997; Tucker et al. 1998; St Amand et al. 2000; Mandler and Neubüser 2001).
The early stages of hair and tooth development are very similar beginning with the thickening of the epithelium to form a placode, which will then bud into the mesenchyme. The hair and tooth epithelia induce the formation of the dermal and dental papillae, respectively. Transient signalling centers form in the placode and at the tip of the bud. The hair bud will then grow to encircle the dermal papilla. Matrix cells in the epithelium contribute to the formation of the hair shaft, and epidermal stem cells reside in the bulge. During cap stage of tooth development the primary enamel knot forms in the epithelial organ. Epithelial cervical loops are sites of active proliferation. Secondary enamel knots are seen at the bell stage directing the shaping of the tooth crown.

Transient epithelial signalling centres are seen first in the placode, and later at the bud stage at the tip of the invaginating bud (Figure 2). Around this time the potential to direct odontogenesis shifts to the mesenchyme. The tooth placode expresses several signalling molecules (Jernvall and Thesleff 2000; Thesleff and Mikkola 2002). Precise functions for all of these in the placode have not been discovered but at least placodal BMP signalling is essential for tooth development. Epithelial BMP4 upregulates the expression of mesenchymal Bmp4 via the transcription factor Msx1, which is required for tooth development (Vainio et al. 1993; Satokata and Maas 1994; Chen et al. 1996). Mesenchymal BMP4 further stimulates Lef1 in the mesenchyme, and Lef1 mutants have no teeth (van Genderen et al. 1994; Kratochwil et al. 1996). Shh is also expressed in the tooth placode, but its role there is not entirely clear. Wnt7a is expressed adjacent to Shh in the oral epithelium. Overexpression of Wnt7b in the ectoderm results in lack of Shh expression and absence of tooth formation (Sarkar et al. 2000). Similarly, inhibition of Shh with antibodies or inhibitors arrests tooth development (Cobourne et al. 2001). On the other hand, transgenic mice overexpressing BMP4 show no detectable Shh expression in the early mandible and nevertheless have no tooth defects (Zhang et al. 2000). It is of course possible that the lack of Shh signalling can be compensated by the downstream effects of BMP overexpression.

At cap stage the signalling centre is called the primary enamel knot, and the cells of the knot can be distinguished histologically by their condensed appearance, and by their lack of proliferation. It has been postulated that mesenchymal signals, such as BMP4, regulate the
formation of the enamel knot. Exogenous BMP4 can induce the expression of two enamel knot markers, Msx2 and p21 (Jernvall et al. 1998; Bei et al. 2000). All in all, the enamel knot expresses specifically at least 15 different signalling factors and transcription factors that regulate either the shaping of the tooth crown or the function of the enamel knot itself (Pispa and Thesleff 2003). One of them, LEF1, has been shown to have one specific function in the knot: to induce Fgf4 (Kratochwil et al. 2002). FGF4 can upregulate mesenchymal FGFs (Kettunen et al. 2000), which are missing in Lef1 mutant teeth (Kratochwil et al. 2002). Mesenchymal FGFs stimulate epithelial proliferation in the epithelial cervical loops (Jernvall et al. 1994; Kettunen et al. 2000). Shh, which is expressed in the enamel knot, affects proliferation of the lingual cervical loop, but conditional inactivation of the Shh receptor, Smoothened, in the epithelium does not inhibit cervical loop growth indicating that the effect on proliferation is achieved via the mesenchymally expressed Smoothened (Dassule et al. 2000; Gritli-Linde et al. 2002). Thus, promotion of epithelial proliferation by Wnt and Shh signalling is regulated by the enamel knot but mediated via the mesenchyme. Subsequently the enamel knot is removed by apoptosis (Jernvall et al. 1994; Vahtokari et al. 1996ab).

At the bell stage new signalling centres, secondary enamel knots, form. Their locations correlate well with the sites of the future cusps, which are the highest peaks of the molar tooth crown (Jernvall et al. 2000). Dental hard tissues, dentin and enamel, will then be deposited by the differentiated mesenchymal odontoblasts and epithelial ameloblasts respectively. Terminal differentiation of odontoblasts and ameloblasts is governed by interactions between the epithelium and mesenchyme (Thesleff and Hurmerinta 1981). The integrity of the extracellular matrix between the epithelium and mesenchyme is also crucial for ameloblast differentiation, for example mutations in laminin 5 disrupt enamel formation (Ryan et al. 1999). Shh is implicated also at this stage since conditional Shh mutants have disrupted enamel (Dassule et al. 2000).

The study of stem cells in teeth is an area of active research, particularly because of the potential clinical applications. The rodent incisor has been a good model to study dental stem cells as it grows continuously. The continuous growth has been attributed to stem cells regulated by FGF10 and Notch signalling (Harada et al. 1999, 2002; Tummers and Thesleff 2003).

1.1.2. Hair development

There is considerable variability in the structure, shape, cycling, and patterning of hairs in different species. Even within one individual several hair types can be found. For example, mouse pelage hair is a mixture of four different types of hairs: guard hairs, awls, auchenens, and zigzags. Zigzags (circa 60-70% of all hairs) and awls (circa 30%) are most numerous, whereas auchenens (circa 5-10%) and guard hairs (2%) are much rarer (Fraser 1951). The hair types can be differentiated from each other by their length, shape, width, and the timing of their development. Guard hair follicles initiate development at E14, awls at E17, and zigzags and auchenens begin to develop in newborn skin (Mann 1962).

Despite the diversity in form all hair follicles develop in a very similar sequence (Figure 2) (Hardy 1992; Millar 2002). A placode forms in the epidermis and buds into the underlying dermis. The base of the bud encircles the mesenchymal dermal papilla. The hair matrix, immediately next to the papilla, is the source of epithelial cells, which will then migrate upward and differentiate into the concentric layers of the mature hair follicle.
Tissue recombination experiments have shown that prior to placode formation only the mesenchyme is capable of directing hair development (the first dermal message) (Kollar 1970; Hardy 1992). When the bud has formed the inductive potential shifts to the epithelium (the epidermal signal), and thereafter back to the mesenchyme (the second dermal message). The epidermal signal will induce the dermal papilla, and the second dermal message enhances epithelial cell proliferation and growth of the hair bud.

The nature of the first dermal message is not known but there is some indication that Wnt signalling may be involved in hair and feather development. *Wnt11* is expressed in the dermis of the chicken feather-forming field prior to placode formation. Signals from the neural tube, specifically Wnt1, can induce feather formation and *Wnt11* expression (Olivera-Martinez et al. 2002). Also β-catenin is transiently found in the chicken dermis prior to placode formation (Noramly et al. 1999). Transgenic mice overexpressing Dickkopf1, an inhibitor of Wnt signalling, have no hairs, teeth or mammary glands (Andl et al. 2002). LEF1 null mice have defects in hair formation and an absence of teeth and mammary glands (van Genderen et al. 1994).

The epithelial placode can be seen as a central unit of ectodermal organogenesis. A reaction-diffusion model has been put forward for hair and feather development that proposes that the initial (mesenchymal) signals are expressed throughout the prospective morphogenetic field (Turing 1952; Koch and Meinhardt 1994; Barsh 1999; Jiang et al. 1999). These signals activate both positive and negative regulators of placode development, which will compete with each other both in the placode epithelium and the underlying mesenchyme. At the site of the placode the activity of the negative regulators is repressed, and placode formation is promoted. However, the negative regulators are free to diffuse to the interfollicular epidermis and repress placode formation there. The mechanisms of reaction-diffusion and lateral inhibition must be exactly integrated to achieve the regular array of forming hair and feather follicles.

Wnts may act as positive regulators of hair placode formation. Overexpression of β-catenin causes ectopic hair follicle formation (Gat et al. 1998), and lack of β-catenin in the epidermis results in lack of hair follicles (Huelsken et al. 2001). In these experiments it is of course not possible to differentiate between the potential role of Wnt as a mesenchymal inducer and as a placode promoter. FGF signalling can also promote placode formation. FGF2 beads induce feather follicle formation (Widelitz et al. 1996), and mice mutant for the FGF receptor Fgfr2b have abnormal hair follicles (Petiot et al. 2003).

BMPs seem to act as placode inhibitors. For example, beads releasing BMP4 on embryonic skin explants inhibit hair follicle formation (Botchkarev et al. 1999). Although BMPs are expressed in the placode itself, their activity within the placode is believed to be negated by BMP inhibitors. Increasing or decreasing Noggin levels has the same effect on follicle formation as decreasing or increasing BMP activity, respectively (Botchkarev et al. 1999, 2002). In addition to BMPs, TGFβ1 also seems to inhibit hair follicle formation. *Tgfb1* null mice have slightly advanced hair follicle development, and beads of TGFβ1 inhibit follicle formation in vitro (Foitzik et al. 1999).

Several genes are known whose mutations affect the structure and shape of hairs (Sundberg 1994; Millar 2002). Nevertheless, little is known about the signalling events regulating terminal differentiation of hair. Wnt signalling appears to be important again at this stage as LEF1 binds to the promoters of the hair-specific keratins and seems to enhance their
expression levels (Zhou et al. 1995; Dunn et al. 1998). BMPs may also be involved, as manipulating BMP activity causes hair shaft differentiation defects (Blessing et al. 1993; Kulessa et al. 2000). In addition, proper regulation of adhesion and extracellular proteins is necessary for hair differentiation as shown by mice mutant for β1 integrin or laminin-10 (Raghavan et al. 2000; Li et al. 2003).

In contrast to mammalian teeth, new hairs are produced throughout life. Each hair follicle goes through a cycle of development, and new follicles are formed from the remains of the old follicle. Each hair cycle consists of three phases: anagen, the active growth phase, catagen, the degenerative phase, and telogen, the resting phase. The continuous formation of new follicles is ensured by the epidermal stem cells, which reside in the bulge halfway along the length of the follicle. These stem cells can migrate down to the hair matrix but they also contribute to the formation of sebaceous glands and epidermis (Fuchs et al. 2001; Alonso and Fuchs 2003). Changes in hair cycle can affect hair length. FGF5 is a negative regulator of the hair cycle as long-haired angora mice have a mutation in the Fgf5 gene (Hebert et al. 1994). Proper regulation of the EGF signalling has been suggested to be involved in the telogen/anagen and anagen/catagen transitions (Mak and Chan 2003). Wnt signalling has been associated with regulating stem cell fate (Merrill et al. 2001; Niemann et al. 2002).

1.2. Ectodermal dysplasias in humans and mice

Ectodermal dysplasias are congenital defects where the development of two or more ectodermal organs is abnormal. There are over 150 clinically defined dysplasias (Pinheiro and Freire-Maia 1994; Priolo and Lagana 2001). Organs most commonly affected are the hair and teeth, which of course are also the easiest to score. Defects include missing or hypoplastic hair follicles of the scalp, eyebrows, and lashes, hypodontia of the teeth, absent or reduced sweating, and hypoplastic nails, and they are occasionally linked with limb defects, immunological aberrations, cleft lip or cleft palate and mental retardation. The genetic and molecular study of these syndromes should identify some of the regulatory molecules common to and possibly specific for ectodermal organs.

The most common of the ectodermal dysplasias is the X-linked hypohidrotic ectodermal dysplasia (HED or EDA; OMIM 305100). It occurs at an estimated incidence of one case per 100 000 births (Clarke 1987). Since it is X-linked the patients are generally male, but the heterozygous carrier females exhibit some symptoms of the disorder. The autosomal forms of HED are symptomatically identical to the X-linked form (Munoz et al. 1997). Diagnosis of patients is often done when the eruption of the primary teeth is delayed in the early childhood. The patients usually have absence of several deciduous and permanent teeth. The hair is sparse and fine and often associated with premature male balding. The most severe symptom that warrants natal diagnosis is the lack of sweat glands, and therefore the risk of hyperthermia. Undiagnosed this symptom is the cause of the 30% early childhood lethality associated with HED. Adult patients suffer from heat intolerance but have no increased mortality rates (Clarke et al. 1987).

Three mouse models exist that mimic HED: Tabby, downless, and crinkled (Falconer et al. 1951; Falconer 1952; Philips 1960; Mouse Genome Database (MGD)). These were first suggested to be the mouse counterparts of the human HEDs by their similar phenotype and by the syntenically similar gene locuses (Blecher 1986). Later the cloning of the genes behind these syndromes confirmed this (I, II; Kere et al. 1996; Ferguson et al. 1997; Headon and Overbeek 1999; Monreal et al. 1999; Headon et al. 2001; Yan et al. 2002).
Tabby/downless/crinkled (Ta/dl/cr) mice may have missing incisors and third molars, and the cusps of their molars are reduced in number (Grüneberg 1965). The first and last waves of hair follicle formation are missing so that the adult mice lack guard hairs and zigzags (Falconer et al. 1951; Falconer 1953; Claxton 1967; Vielkind and Hardy 1996; Laurikkala et al. 2002). Several glands are missing, small, or abnormally shaped. These include the sweat glands (normally only in the palms of mouse paws), lacrimal and Meibomian glands, and submandibular salivary gland (Grüneberg 1971; Blecher et al. 1983).

Thirteen of the genes responsible for ectodermal dysplasias have been identified during the last few years (Table 1). Five of them are associated with the tumor necrosis factor (TNF) pathway (see results and reviews by Mikkola and Thesleff 2003 and Smahi et al. 2002). The rest include the adhesion molecules plakophilin-1 (McGrath et al. 1997), nectin-1 (Suzuki et al. 2000), and connexin-30 (Lamartine et al. 2000), the transcription factor p63 (Celli et al. 1999) and the structural molecules keratins 6A, 6B, 16 and 17 (Bowden et al. 1995; McLean et al. 1995; Smith et al. 1998).

Table 1. Mutated genes in ectodermal dysplasias

<table>
<thead>
<tr>
<th>Gene</th>
<th>Type of protein</th>
<th>Ectodermal dysplasia</th>
<th>OMIM no</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eda (ectodysplasin)</td>
<td>TNF superfamily member</td>
<td>X-linked HED or EDA</td>
<td>305100</td>
<td>Kere et al. 1996</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Bayés et al. 1998</td>
</tr>
<tr>
<td>Edar</td>
<td>TNFR superfamily member</td>
<td>Autosomal recessive HED</td>
<td>224900</td>
<td>Monreal et al. 1999</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Autosomal dominant HED</td>
<td>129490</td>
<td>Monreal et al. 1999</td>
</tr>
<tr>
<td>Edaradd</td>
<td>DD containing adaptor protein</td>
<td>Autosomal recessive HED</td>
<td>224900</td>
<td>Headon and Overbeek 2001</td>
</tr>
<tr>
<td>IkBα</td>
<td>Inhibitor of NFκB</td>
<td>EDA-ID</td>
<td></td>
<td>Courtois et al. 2003</td>
</tr>
<tr>
<td>IKKγ or NEMO</td>
<td>Subunit of IKK</td>
<td>Incontinentia pigmenti</td>
<td>308300</td>
<td>Smahi et al. 2000</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>OL-EDA-ID</td>
<td>300301</td>
<td>Döffinger et al. 2001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HED-ID</td>
<td>300291</td>
<td>Zonana et al. 2000</td>
</tr>
<tr>
<td>Plakophilin 1</td>
<td>Cytoplasmic desmosomal plaque</td>
<td>Ectodermal dysplasia/skin fragility syndrome</td>
<td>604536</td>
<td>McGrath et al. 1997</td>
</tr>
<tr>
<td>Nectin-1</td>
<td>Adherens junction protein</td>
<td>ED4 (Margarita Island type)</td>
<td>225060</td>
<td>Suzuki et al. 2000</td>
</tr>
<tr>
<td>Connexin-30</td>
<td>Gap junction component</td>
<td>Clouston syndrome</td>
<td>129500</td>
<td>Lamartine et al. 2000</td>
</tr>
<tr>
<td>p63</td>
<td>Transcription factor</td>
<td>EEC3</td>
<td>604292</td>
<td>Celli et al. 1999</td>
</tr>
<tr>
<td>Keratins 6A, 6B</td>
<td>Structural filamentous</td>
<td>Pachyonychia congenita</td>
<td>167200</td>
<td>Bowden et al. 1995</td>
</tr>
<tr>
<td>Keratins 16, 17</td>
<td>proteins</td>
<td>LII</td>
<td>167210</td>
<td>McLean et al. 1995</td>
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<td></td>
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<td>Smith et al. 1998</td>
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</table>
1.3. Tumor necrosis factor (TNF) signalling

1.3.1. TNF superfamily

The first TNF molecule isolated, TNFa, was characterised by its ability to kill tumour cells, hence its name “tumor necrosis factor” (Pennica et al. 1984). Thereafter TNFa homologues that do not all share the tumour cell killing ability have been isolated. The TNF superfamily currently consists of 19 TNF-related ligands, listed in http://www-personal.umich.edu/~ino/List/996.htm (Locksley et al. 2001; Bodmer et al. 2002). They are type II membrane proteins (the N-terminus is intracellular) and consist of four domains: the intracellular part, the transmembrane domain, the stalk region, and the conserved TNF domain. The intracellular portion is generally short (10-80 aa) and does not contain any recognisable protein domains (Bodmer et al. 2002). Although it has been suggested that some TNF ligands can “reverse signal” back to the cell which expresses them (Cayabyab et al. 1994; van Essen et al. 1995; Wiley et al. 1996; Orengo et al. 1997) in general the intracellular domain is not believed to have any signalling activity. The extracellular circa 150 aa long TNF domain is composed of anti-parallel β-strands and is responsible for trimer formation and receptor binding (Banner et al. 1993; Cha et al. 2000; Hymowitz et al. 2000; Bodmer et al. 2002). Generally, the trimers are homotrimers, but one exception is known: lymphotoxin-α (LTα) can form heterotrimers with LTβ (Browning et al. 1993). Between the transmembrane domain and the TNF domain lays a stalk area (Bodmer et al. 2002). Some TNF ligands are cleaved at the stalk region by proteases such as furin and shed from the plasma membrane (Orlinick and Chao 1998). This cleavage is required for a functional protein in some cases (Chen et al. 2001; Elomaa et al. 2001; Schneider et al. 2001), but may also be used as a means of inhibiting receptor binding and subsequent signalling (Tanaka et al. 1998). TNF ligands and members of the C1q family show structural homology indicating an evolutionary link (Shapiro and Scherer 1998; Bodmer et al. 2002).

1.3.2. TNF receptors

The TNF receptor (TNFR) superfamily has currently 32 members that are mostly type I membrane proteins (their N-terminus resides outside the cell) (Bodmer et al. 2002; Schneider et al. 2003). They are listed in http://www-personal.umich.edu/~ino/List/996.htm. The cytoplasmic C-terminus has no known enzyme activity. Instead, it can bind cytoplasmic adapter proteins and participate in signal transduction inside the cell (Orlinick and Chao 1998; Locksley et al. 2001). The extracellular part of TNF receptors contains a variable number of circa 40 aa cysteine-rich domains that are responsible for ligand binding (Orlinick and Chao 1998; Bodmer et al. 2002). Binding of trimeric TNF ligands has been believed to induce clustering of the receptors into trimers and subsequent signal transduction (see references in Orlinick and Chao 1998). Current evidence suggests though that self-assembly of receptor trimers is required for ligand binding (Chan et al. 2000; Siegel et al. 2000). Some TNF ligand/receptor pairs are specific for each other but differential binding also occurs. For example, the TRAIL ligand can bind to five different receptors and conversely TNFR1 can bind both TNFa and LTα (Bodmer et al. 2002).

Some of the receptors are in soluble or membrane attached form that lack the intracellular part (Bodmer et al. 2002). They are believed to act as decoy receptors inhibiting the ligand interaction with the functional receptor (Gatanaga et al. 1990; Lantz et al. 1990; Seckinger et al. 1990; Simonet et al. 1997; McDermott et al. 1999; Schneider et al. 2003). Regulation of the decoy receptor expression may be one means to regulate TNF signalling.
1.3.3. Intracellular signal transduction by TNFs

The intracellular signalling network downstream of TNF receptors is complex (Figure 3). It consists of a range of adapter proteins and kinases that are used in different combinations (Aggarwal 2000; Dempsey et al. 2003). Some of the signal mediators are also utilised by other ligands and receptors, for example by the Toll/interleukin-1 receptor family (Toll/IL-1R) (Cao et al. 1996; Ye et al. 2002). The main cellular responses to TNF signalling include apoptosis and the activation of the transcription factors nuclear factor κB (NF-κB) and activator protein-1 (AP-1) (Dempsey et al. 2003). These transcription factors will then translocate into the nucleus and induce (or repress) their respective target genes. Which response occurs depends on the ligand, receptor and the cellular context.

Figure 3. A simplistic view of some of the TNF signal transduction machinery.

Figure 3. A simplistic view of some of the TNF signal transduction machinery. Trimeric TNF ligands bind to their trimeric membrane receptors, TNFRs. The signal is mediated by DD-containing adapter proteins, such as FADD, Edaradd, and TRADD, and also TRAF-proteins. Intracellular molecules, e.g. A20 and c-Src, modify the signal transduction pathway either positively or negatively. Kinases such as IKK, JNK, and p38 are required for phosphorylation events ensuring the nuclear entry of transcription factors NF-κB and AP-1. IκB is an inhibitor of NF-κB and its phosphorylation and subsequent degradation is necessary for NF-κB translocation to the nucleus. Final outcomes of TNF signalling include apoptosis and transcriptional activation/repression of NF-κB and AP-1 target genes.

The intracellular domains of TNF receptors contain potential binding sites for interactions with cytosolic proteins (Locksley et al. 2001; Dempsey et al. 2003). The best characterised of these sites is the circa 80 aa long death domain (DD) that was first identified because of its association with promotion of apoptosis (Tartaglia et al. 1993). The DD domain binds adapter proteins such as FADD, TRADD, and Edaradd that share the DD domain. Not all DDs are created equal as TNFRs specifically bind certain adapter proteins, e.g. Edar binds only Edaradd (Headon et al. 2001; Yan et al. 2002). TRADD, Edaradd and in some circumstances FADD can activate NF-κB (Hsu et al. 1995; Hu et al. 2000; Headon et al. 2001; Yan et al. 2002). FADD and also TRADD can promote apoptosis (Hsu et al. 1995; Kischkel et al. 1995). In addition, TRADD activates the Jun N-terminal kinase (JNK), which is a known activator of AP-1 (Eliopoulos et al. 1999).

The receptors can also interact with TNF receptor-associated factors (TRAFs) (Inoue et al. 2000; Chung et al. 2002). The interaction can be direct as has been shown by crystallography studies of a CD40 receptor trimer forming a complex with three TRAF molecules (McWhirter et al. 1999). The interaction can also be indirect so that another protein links both the TNFR and the TRAF molecules (Hsu et al. 1996; Shu et al. 1996; Headon et al. 2001; Yan et al. 2002). TRAFs have been associated with both the NF-κB and the AP-1 pathways (Inoue et al.
In mammals six TRAFs are known. They are expressed in differential patterns, and have potentially different biological functions (Chung et al. 2002).

A number of other molecules have been found associated with TNF receptors or with proteins interacting with them. The function or importance of many of them is not known, but some have been shown to regulate TNF signalling either positively or negatively (Ledgerwood et al. 1999; Aggarwal 2000). For example, A20 is a zinc finger protein that inhibits TNFα induced apoptosis by disrupting apoptotic adapter protein recruitment to the receptor (He and Ting 2002). At the same time, NF-κB signalling induces its expression (Opipari et al. 1992; Sarma et al. 1995). Activation of NF-κB has been shown to protect cells from TNF-induced apoptosis (Van Antwerp et al. 1998), and the upregulation of A20 may be one potential mechanism for this.

Intracellular kinases are required for activation of the NF-κB and AP-1 transcription factors. The main kinases downstream of TNF signalling are the IκB kinase (IKK), and the mitogen-activated protein kinases such as Jun N-terminal kinase (JNK) and p38 kinase. In general, IKK activates NF-κB (Israël 2000), and JNK and p38 kinases AP-1 (Shaulian and Karin 2002). The IKK complex consists of a tetramer (or more specifically two dimers) of IKKγ (or NEMO) subunits, and two homo- or heterodimers of IKKα and IKKβ (or IKK1 and IKK2), the kinase components (Tegethoff et al. 2003). The precise mechanism of how the IKK complex is activated is not clear (Israël 2000). A recent report has implicated ubiquitination of IKKγ as a potential regulative method (Tang et al. 2003). In addition, NF-κB inducing kinase (NIK) seems to be involved in specific activation of IKKα (Regnier et al. 1997; Ling et al. 1998; Senftleben et al. 2001). c-Src tyrosine kinase has been suggested as one way of activating IKKβ (Huang et al. 2003). Indeed, a TNFR, RANK, can activate c-Src (Wong et al. 1999), and mice lacking c-Src or expressing a dominant negative Src have a similar phenotype to RANK null mice (Soriano et al. 1991; Xing et al. 2001). JNK and p38 are activated by phosphorylation by a series of MAP kinases (Mindn and Karin 1997).

The NF-κB transcription factor is a homo- or heterodimer consisting of proteins of the Rel family (relA or p65, relB, c-rel, p50 or NF-κB1, p52 or NF-κB2). p50 and p52 are processed from inactive precursors p105 and p100, respectively (Ghosh et al. 1998; Ghosh and Karin 2002). In the cytosol NF-κB dimer binds to its inhibitor, one of the IκB family members. When IκB is degraded, NF-κB is free to translocate from the cytoplasm to the nucleus and bind to the promoters of its target genes. The degradation of IκB is accomplished by its phosphorylation by the IKK complex (Israël 2000; Ghosh and Karin 2002). IκB degradation has been studied to a large degree, and is seen as the canonical NF-κB activation pathway. IKKα and IKKβ can both phosphorylate IκB. Analysis of IKKα and IKKβ mutant phenotypes has revealed though that the two kinases may compensate for each other in some contexts, but they also have more specific functions (Hu et al. 1999b; Li et al. 1999a,b,c; Takeda et al. 1999; Tanaka et al. 1999; Pasparakis et al. 2002a,b). Although IKKα seems to act via IκB degradation during mammary gland differentiation (Cao et al. 2001) it can also activate NF-κB by an alternative pathway. IKKα mediated phosphorylation of the inactive precursor, p100, causes p100 proteolysis into the functional subunit, p52 (Senftleben et al. 2001). In the epidermis it also seems to be able to function in an NF-κB-independent manner (Hu et al. 2001). An additional level of regulation of NF-κB activity has been postulated to be caused by the phosphorylation of NF-κB subunits (Schulze-Osthoff et al. 1997; Schmitz et al. 2001; Ghosh and Karin 2002).
AP-1 is a homo- or heterodimer composed of members of the Jun, Fos, Maf, or ATF family. These proteins contain the bZIP DNA binding motif (Jochum et al. 2001; Shaulian and Karin 2002). The regulation of AP-1 activity can occur at several levels. Phosphorylation or other post-translational modifications can affect protein activity or stability. Transcriptional regulation of AP-1 mRNA levels controls the amount of protein available (Karin et al. 1997; Shaulian and Karin 2002). TNF stimulated JNK activity seems to affect both protein stability and transcriptional regulation (Ventura et al. 2003).

TNF signalling may also interact with other signalling pathways. Tcf/β-catenin signalling is required for induction of cyclin D1 by IKKα (Albanese et al. 2003), and glycogen synthase kinase-3b (GSK3b), a negative regulator of the Wnt pathway, affects the stability of an NF-κB precursor (Demarchi et al. 2003). In the chick limb NF-κB has been suggested to mediate FGF signalling (Bushdid et al. 2001). A synergistic interaction between TNFα signalling and EGFR has also been proposed (Hirota et al. 2001).

### 1.3.4. The role of TNF signalling

Since the first TNF found, TNFα, could enhance apoptosis of cancerous cells, it raised hopes of its use as a therapeutic agent in the treatment of cancer. Further studies have shown though that the downstream effects of TNF signalling include not only apoptosis, but also cellular proliferation, differentiation, migration (Benoliel et al. 1997; Locksley et al. 2001) and even protection from apoptosis (Van Antwerp et al. 1998). The use of TNFα in cancer therapy was hindered by its toxic, mainly inflammatory, effects. Indeed, the majority of TNF signalling is related to inflammation and to the immune system (Table 2; Pfeffer 2003). Knockouts of many of the TNF/TNFRs have impaired immune response or defects in secondary lymphoid organs. Of the known TNF ligands, at least 13 are associated with T or B cell activation. Although no TNF/TNFR homologous genes have been described in yeast and Caernohabditis elegans, Drosophila melanogaster has one TNF and one TNF receptor homologue. The ligand, Eiger, induces JNK-mediated apoptosis, and has been shown to interact physically with the receptor, Wengen (Igaki et al. 2002; Kanda et al. 2002; Moreno et al. 2002). The homologue of NF-κB in flies, dorsal, is a key component in the determination of dorsal-ventral polarity and of the innate immune response, but it is activated by a Toll receptor rather than TNFRs (Belvin and Anderson 1996).

In vertebrates some of the TNF signalling has more developmental roles (Table 2). The ligand RANKL, and its receptor RANK are required for osteoclast differentiation (Dougall et al. 1999; Hsu et al. 1999; Kong et al. 1999; Suda et al. 1999). Lack of either one causes osteopetrosis in transgenic mice. The balance of RANK signalling is controlled at least partially by expression of a dominant-negative decoy receptor, osteoprotegerin (OPG) (Simonet et al. 1997; Tsuda et al. 1997). An additional function for RANK has been discovered also in the later stages of mammary gland differentiation. In RANKL or RANK null mice the mammary gland develops up to puberty but does not differentiate normally during pregnancy (Fata et al. 2000). This is due to the lack of NF-κB activation, and specifically the lack of the target of NF-κB in mammary gland, cyclin D, which is required for proliferation of mammary epithelial cells (Brantley et al. 2001; Cao et al. 2001). OPG also possibly affects the vascular system as the presence of OPG in the media of great arteries is believed to protect against arterial calcification (Min et al. 2000; Schoppet et al. 2002). Similarly, in endothelial cell cultures OPG prevents apoptosis (Malyankar et al. 2000). In the chick limb inhibition of NF-κB activity results in a dysmorphic apical ectodermal ridge (AER) (Bushdid et al. 1998;
Kanegae et al. 1998). However, it has been proposed that NF-κB is activated indirectly by FGF receptors rather than by TNFRs (Bushdid et al. 2001).

NF-κB has also been associated with skin development. Transgenic mice overexpressing NF-κB subunits have a hypoproliferative epidermis (Seitz et al. 1998). Conversely overexpression of IkBα results in epidermal hyperproliferation (Seitz et al. 1998; van Hogerlinden et al. 1999). IkBα null mutant mice also have defective formation of the epidermis (Beg et al. 1995; Klement et al. 1996). It is unclear though whether NF-κB signalling is directly involved in epidermal proliferation or whether the defects seen are secondary and due to an inflammatory response (Kaufman and Fuchs 2000; Pasparakis et al. 2002a). IKKα is required for differentiation of the epidermis but as already mentioned this seems to occur by an NF-κB-independent mechanism (Hu et al. 1999b; Li et al. 1999a; Takeda et al. 1999; Hu et al. 2001). The role of TNF signalling in ectodermal organ development has recently been discovered (for review see Mikkola and Thesleff 2003), and will be discussed in more detail in the following results and discussion.

Table 2. Phenotypes of mice lacking TNF/TNFR superfamily genes

<table>
<thead>
<tr>
<th>Ligands</th>
<th>Defects of the immune system</th>
<th>Other defects</th>
<th>Corresponding human disease/OMIM number</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα</td>
<td>B-cell areas in spleen</td>
<td></td>
<td></td>
<td>Pasparakis et al. 1996</td>
</tr>
<tr>
<td>LTα</td>
<td>Peripheral lymphoid organs missing</td>
<td></td>
<td></td>
<td>De Togni et al. 1994, Banks et al. 1995</td>
</tr>
<tr>
<td>LTβ</td>
<td>Peripheral lymphoid organs missing</td>
<td></td>
<td></td>
<td>Koni et al. 1997</td>
</tr>
<tr>
<td>FasL</td>
<td>Impaired T-cell death</td>
<td>Mammary gland involution defect</td>
<td>Autoimmune lymphoproliferative syndrome / 601859</td>
<td>Takahashi et al. 1994, Song et al. 2000</td>
</tr>
<tr>
<td>CD40L</td>
<td>B-cell differentiation defective</td>
<td>Possible association with Alzheimer's disease</td>
<td>Immunodeficiency with hyper-IgM / 308230</td>
<td>Xu et al. 1994, Tan et al. 2002</td>
</tr>
<tr>
<td>OX40L</td>
<td>Defective T-cell response</td>
<td></td>
<td></td>
<td>Murata et al. 2000</td>
</tr>
<tr>
<td>4-1BBL</td>
<td>Defective T-cell response, susceptible to infection</td>
<td></td>
<td></td>
<td>DeBenedette et al. 1999</td>
</tr>
<tr>
<td>TRAIL</td>
<td>Impaired T-cell death</td>
<td>Susceptible to tumor metastasis</td>
<td></td>
<td>Cretney et al. 2002, Lamhamedi-Cherradi et al. 2003</td>
</tr>
<tr>
<td>BAFF</td>
<td>Required for B-cell development</td>
<td></td>
<td></td>
<td>Schiemann et al. 2001</td>
</tr>
<tr>
<td>LIGHT</td>
<td>Impairment of T-cell function</td>
<td></td>
<td></td>
<td>Tamada et al. 2002</td>
</tr>
<tr>
<td>Ectodysplasin</td>
<td>-</td>
<td>Ectodermal dysplasia</td>
<td>X-linked HED or EDA / 305100</td>
<td>Falconer 1952</td>
</tr>
<tr>
<td>RANKL</td>
<td>T- and B-cell differentiation defect, missing lymph nodes</td>
<td>Osteopetrosis</td>
<td>Mammary gland differentiation</td>
<td>Kong et al. 1999, Fata et al. 2000</td>
</tr>
</tbody>
</table>
2. AIMS OF THE STUDY

In order to study the early common aspects of ectodermal organogenesis this thesis work focused on the analysis of the natural mouse mutant *Tabby*, which was known to affect the development of several ectodermal organs. Specifically the aims were:

- to clone and characterise the *Tabby* gene.

- to analyse the molecular pathogenesis of the phenotype of the *Tabby* teeth.

- to study the expression patterns of *ectodysplasin* (*Tabby*), its receptor *Edar*, and a related TNF receptor *TNFRSF19* during mouse embryogenesis.

- to study the downstream effects of Edar signalling by misexpression of Edar in transgenic mice.
3. MATERIALS AND METHODS

3.1. Mouse strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Used in article</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMRI or CBAT6T6 x NMRI</td>
<td>I,II,III,IV</td>
<td>mRNA expression studies</td>
</tr>
<tr>
<td><em>Tabby</em> (B6CBACa-A&lt;sup&gt;−&lt;/sup&gt;/A Ta/0 Jackson Laboratory, Bar Harbor, USA)</td>
<td>I,II,III</td>
<td>Analysis of <em>Tabby</em> phenotype</td>
</tr>
<tr>
<td>FVB</td>
<td>V</td>
<td>Control strain for K14-<em>Edar</em> mice</td>
</tr>
<tr>
<td>K14-<em>Edar</em></td>
<td>V</td>
<td>Misexpression of <em>Edar</em> in the ectoderm under the keratin 14 promoter</td>
</tr>
</tbody>
</table>

Generation of Ta-βgal mice

Vector pBGβgal was constructed by inserting BgIII-Xbal excised beta-galactosidase gene from pTKβ (Clontech) into BamHI-Xbal sites of pBS SK+ (Stratagene). A blunted Ncol-Smal fragment from the mouse ectodysplasin promoter (nt 272-2517, GenBank accession number Y13438) was excised from plasmid pGL3-Tabby and cloned into the blunted XhoI site of pBGβgal. The transgenic construct was released from the vector by partial digestion with KpnI and SacI, and microinjected into the pronucleus of FVB mouse fertilized oocytes. Transgenic animals were identified by PCR using primers 5'-ACCTCCTCCCTCATCCCTTCTC-3' for ectodysplasin promoter and 5'-AGACCAATGCCTCCCAGACC-3' for beta-galactosidase gene. Four founders were obtained of which three exhibited LacZ staining in a nearly identical manner and one did not express beta-galactosidase.

3.2. Ectodysplasin clones

<table>
<thead>
<tr>
<th>cDNA clones</th>
<th>Source</th>
<th>Description</th>
<th>Article</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ta-A1</td>
<td>Mouse E17 5' stretch cDNA library (Clontech)</td>
<td>Ectodysplasin-A1 partial cDNA (3995 bp) subcloned into pCR2.1 (Invitrogen). Complete cDNA sequence, AF016628 (GenBank), was obtained by combining with 3’ sequence of clone D85 (Research Genetics, USA).</td>
<td>I</td>
</tr>
<tr>
<td>Ta-A2</td>
<td>Mouse E17.5 cDNA library (Clontech)</td>
<td>Ectodysplasin-A2 cDNA (4937 bp) subcloned into pGEM5Z (Promega). EMBL accession number AJ243657.</td>
<td>II</td>
</tr>
<tr>
<td>Ta-A3</td>
<td>Mouse E17.5 cDNA library (Clontech)</td>
<td>Ectodysplasin-A2 cDNA (3096 bp) subcloned into pGEM5Z (Promega). EMBL accession number AJ243658.</td>
<td>II</td>
</tr>
<tr>
<td>Ta-B</td>
<td>Mouse E17 5' stretch cDNA library (Clontech)</td>
<td>Ectodysplasin-B cDNA (1879 bp) subcloned into pCR2.1 (Invitrogen). GenBank accession number AF016630.</td>
<td>I</td>
</tr>
<tr>
<td>Ta-C</td>
<td>Mouse E17 5' stretch cDNA library (Clontech)</td>
<td>Ectodysplasin-C cDNA (1562 bp) subcloned into pCR2.1 (Invitrogen). GenBank accession number AF016631.</td>
<td>I</td>
</tr>
</tbody>
</table>
### 3.3. Probes

The following probes were used for in situ hybridisation:

<table>
<thead>
<tr>
<th>Probe</th>
<th>Description or reference</th>
<th>Used in:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ectodysplasin pTaNoBg</td>
<td>398 bp ectodysplasin cDNA fragment. Described in I. Recognizes all ectodysplasin splice forms.</td>
<td>I,II,III</td>
</tr>
<tr>
<td>Ectodysplasin PTE2A</td>
<td>793 bp ectodysplasin-A3 3’UTR fragment. Described in II. Recognizes all ectodysplasin-A splice forms.</td>
<td>I,II</td>
</tr>
<tr>
<td>Ectodysplasin pTaB</td>
<td>806 bp ectodysplasin-B cDNA fragment. Described in II.</td>
<td>II</td>
</tr>
<tr>
<td>Ectodysplasin pTaNeSp</td>
<td>Laurikka et al. 2001 (1836 bp). Recognizes all ectodysplasin splice forms.</td>
<td>IV</td>
</tr>
<tr>
<td>Edar PTOPOdl 4/3</td>
<td>1224 bp Edar cDNA. Described in IV.</td>
<td>IV</td>
</tr>
<tr>
<td>Edar PTOPOdl 3</td>
<td>Laurikka et al. 2001 (1415 bp)</td>
<td>V</td>
</tr>
<tr>
<td>Bmp2</td>
<td>Vainio et al. 1993</td>
<td>III</td>
</tr>
<tr>
<td>Bmp4</td>
<td>Vainio et al. 1993</td>
<td>III,V</td>
</tr>
<tr>
<td>Egfr</td>
<td>Murine cytoplasmic domain of Egfr cDNA in pCRTMII (Invitrogen). Gift of David C. Lee.</td>
<td>III</td>
</tr>
<tr>
<td>Fgf3</td>
<td>Wilkinson et al. 1988</td>
<td>III</td>
</tr>
<tr>
<td>Fgf4</td>
<td>Jernvall et al. 1994</td>
<td>III</td>
</tr>
<tr>
<td>Fgf8</td>
<td>Heikinheimo et al. 1994</td>
<td>III</td>
</tr>
<tr>
<td>Fgf10</td>
<td>Bellusci et al. 1997</td>
<td>III</td>
</tr>
<tr>
<td>Lef1</td>
<td>Travis et al. 1991</td>
<td>V</td>
</tr>
<tr>
<td>L-fng</td>
<td>Harada et al. 1999</td>
<td>III</td>
</tr>
<tr>
<td>Patched</td>
<td>Kim et al. 1998</td>
<td>V</td>
</tr>
<tr>
<td>p21</td>
<td>740 bp fragment of murine cDNA. Gift of Bert Vogelstein.</td>
<td>V</td>
</tr>
<tr>
<td>Shh</td>
<td>Vaahktokari et al. 1998a</td>
<td>III</td>
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<tr>
<td>TNFRSF19</td>
<td>365 bp PCR fragment from murine TNFRSF19 (nt 1374-1738, GenBank accession number AB040432) cloned into pCRII-TOPO (Invitrogen)</td>
<td>IV</td>
</tr>
<tr>
<td>Wnt10a</td>
<td>Dassule and McMahon 1998</td>
<td>III</td>
</tr>
</tbody>
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### 3.4.1. Methods used in articles I-V

<table>
<thead>
<tr>
<th>Method</th>
<th>Article</th>
</tr>
</thead>
<tbody>
<tr>
<td>Generation and analysis of K14-Edar mice</td>
<td>V</td>
</tr>
<tr>
<td>Screening of genomic and cDNA libraries, cloning, and sequencing</td>
<td>I,II</td>
</tr>
<tr>
<td>Tissue culture and bead experiments</td>
<td>III</td>
</tr>
<tr>
<td>Analysis of adult mouse tooth phenotype (skeletal preparations)</td>
<td>III</td>
</tr>
<tr>
<td>Histology</td>
<td>I,II,III,IV,V</td>
</tr>
<tr>
<td>Radioactive in situ hybridisation on sections</td>
<td>I,II,III,IV,V</td>
</tr>
<tr>
<td>Whole mount in situ hybridisation</td>
<td>III,IV,V</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td>III</td>
</tr>
</tbody>
</table>

### 3.4.2. LacZ staining

LacZ staining was performed essentially as in standard protocols using 2% paraformaldehyde and 0.2% glutaraldehyde as fixative, either on whole mount tissues or on 7 μm cryosections. Cryosections were counterstained with Nuclear Fast Red.
4. RESULTS AND DISCUSSION

4.1. Cloning of the Tabby gene (I, II)

The human X-linked HED gene was positional cloned in 1996 (Kere et al. 1996) but at the time only one cDNA was isolated. The coding sequence corresponded to a short 135 aa type II membrane protein. The extracellular portion contained four Gly-X-X repeats reminiscent of collagens but no other functional domains. Based on these results two strategies were adopted in cloning the mouse homologous gene, Tabby, from a mouse E17 embryo cDNA library (Clontech). In the first strategy the full-length human cDNA was used as a probe, and five cDNAs were recovered. These corresponded to three different splice forms (Ta-A1, Ta-B, Ta-C). The human EDA-B splice form was also cloned. In the second strategy the mouse genomic region was first recovered by probing a mouse genomic library with a 120 bp PCR fragment obtained from mouse DNA with primers specific for human EDA intron 1 sequence (nt 4497-4617 of GenBank accession number U59227). Seven positive lambda clones were recovered and one (G2) was subcloned into plasmids (pTEX2, pTEX3, pTEX6). The plasmid clones obtained were sequenced and together with sequence obtained from PCR of G2 the sequence was compiled into a contig of 3560 bp. An exon homologous to the human exon 1 was identified. The 1.5 kb insert from pTEX6 including this exon was used as a probe for screening an embryonic cDNA library. Two novel splice forms were obtained (Ta-A2, Ta-A3).

Simultaneously with the cloning of these Tabby splice forms, the cloning of yet another splice form (Ta-A4) was reported (Ferguson et al. 1997). All in all, six different splice variants have been recovered (Figure 4a), none corresponding to the originally isolated human cDNA, which was named EDA-O. Human cDNAs complementary to mouse cDNAs (EDA-A1, EDA-A2, EDA-C) have since been discovered together with three novel uniquely human splice forms (Figure 4b) (Bayés et al. 1998).

Analysis of the open reading frames (ORF) of all splice forms (mouse and human) showed that the N-terminus including a short circa 40 aa intracellular domain, a circa 20 aa transmembrane domain, and 71 aa extracellular sequence was shared between all splice forms, and was coded by one exon. The configuration was predicted to a type II membrane protein. The protein product was named ectodysplasin. A special feature of all the Ta-A splice forms was that they contained in their C-terminus an interrupted stretch of 19 Gly-X-X repeats and a large TNF domain (Figure 5). A furin cleavage site has subsequently been identified between the transmembrane domain and the Gly-X-X repeats (Pääkkönen et al. 2001), and proteolytic cleavage has been shown to be required for protein activity (Chen et al. 2001; Elomaa et al. 2001; Schneider et al. 2001). The TNF domain was found to be most homologous to the TNF ligands APRIL, TRAIL, and lymphotoxin-α (LT-α) (26% identity). Homology to the TNF superfamily was also confirmed by others (Copley 1999; Ezer et al. 1999). The four Ta-A forms differ from each other by small 2-14 aa deletions in the TNF domain. These deletions have been shown to change the receptor-binding properties of the ligands (see below). The function of the shorter splice forms that do not contain a TNF domain or Gly-X-X repeats has not been determined. Expression of Ta-B mRNA was examined with in situ hybridisation in E7 embryos and in the embryonic brain but no signal was recovered. In Northern analysis the main transcript is 5.5-6.0 kb long but a shorter mRNA has been seen in adult testes (2.4 kb).
Figure 4. Murine and human EDA (ectodysplasin) splice forms.

Six Tabby (A) and eight human Eda (B) mRNA isoforms have been discovered. Numbers refer to amino acids. COOH, carboxyterminus; COL, collagen-like Gly-X-X repeats; NH₂, aminoterminus; TM, transmembrane domain; TNF-domain, tumor necrosis factor homology domain. Deletions in the TNF-domain of the A2-A4 forms are indicated with black bars. Hatched box refers to transcript derived from intron 1.

Figure 5. Ectodysplasin is a TNF ligand.

The N-terminus of ectodysplasin is located intracellularly. The extracellular part contains a furin cleavage site, a collagen-like domain (COL), and a TNF domain.
At the same time the genes for the autosomal HED and the *downless* mouse mutant were identified and found to code for a member of the TNF receptor superfamily (Headon and Overbeek 1999; Monreal et al. 1999). The 448 aa protein was named Edar for EDA-receptor. Like many TNF receptors it contains a death domain in its intracellular domain. Studies of ligand binding have shown that Edar specifically binds the ectodysplasin-A1 isoform (Tucker et al. 2000; Yan et al. 2000; Elomaa et al. 2001; Kumar et al. 2001). Interestingly, the ectodysplasin-A2 isoform is incapable of binding Edar due to a mere two aa deletion difference in the TNF domain. Instead, it has been shown to bind to a novel TNF receptor, Xedar (for X-chromosome located Edar homologue) (Yan et al. 2000). The binding affinities of the ectodysplasin-A3 or –A4 splice forms have not been tested. The corresponding human cDNAs have not been isolated which might indicate that these isoforms are aberrant splicing events rather than mRNAs coding for functional proteins. Indeed, the A4 splice form does lack residues conserved in the TNF domain (Ferguson et al. 1997). Mutations found in ectodermal dysplasia patients affect either all splice forms or all the ectodysplasin-A forms (Pääkkönen et al. 2001; Schneider et al. 2001; Vincent et al. 2001; Drögemüller et al. 2002). One exception is an intronic mutation that is predicted, but not shown, to specifically affect ectodysplasin-A1 splicing (Schneider et al. 2001). In summary, it can be concluded that the ectodysplasin-A1 – Edar signalling pathway is responsible for the ectodermal dysplasia phenotype in mice and humans. The importance of ectodysplasin-A2 –Xedar signalling is unclear. Xedar is expressed at later stages of hair follicle formation, and the A2 protein isoform also has been localised at later stages using a labelled Xedar-fusion protein for detection (Yan et al. 2000). Unlike ectodysplasin-A1, neither ectopic expression of the ectodysplasin-A2 isoform in transgenic mice nor prenatal treatment with ectodysplasin-A2 protein effected development or rescued the *Tabby* phenotype (Gaide and Schneider 2003; Mustonen et al. 2003).

### 4.2. Analysis of *Tabby* tooth phenotype and partial rescue by FGF (III)

The *Tabby* tooth phenotype is strongly influenced by the genotype or the mouse background strain (Grüneberg 1965). The dental phenotype of our *Tabby* strain, which was in a B6CBA hybrid background, was analysed first morphologically. 23% of the mice had missing incisors (N=26) and third molars were missing in 17% of half-mandibles (N=52). The cusp number of the first lower molar was reduced to an average of 3.8 cusps in contrast to the seven cusps of wild type mice. *Tabby* tooth development was also studied in in vitro cultures started at E13 and E14. Although the in vitro cusp numbers achieved were lower than in vivo (seven wild type in vivo cusps compared to five in vitro), *Tabby* molars developed in vitro comparably to in vivo as the first molar cusp number was reduced (three cusps on average). The mesio-distal length of the first molar tooth germ was one-third shorter than wild type tooth germs. Peterkova and colleagues have subsequently studied *Tabby* tooth germs using three-dimensional reconstructions of histological sections (Peterkova et al. 2002). They have observed a range of different *Tabby* morphotypes. Although the mesio-distal length of the entire dental epithelium in their study was similar to wild type mice, the first molar (or the most mesial tooth) primordia were consistently shorter agreeing with the in vitro data in article II.

Injections of EGF into neonate *Tabby* mice have been shown to induce sweat gland formation (Blecher et al. 1990). In order to test whether EGF could also rescue the tooth defect of *Tabby* mice, *Tabby* molars were cultured in vitro together with EGF in the medium. This had no effect on *Tabby* tooth development. Instead, addition of FGF10 to media increased *Tabby* cusp number to 3.6 at E13 (compared to 2.8 without FGF10) and to 4.3 at E14 (compared to...
3.4 without FGF10). FGF10 is normally expressed in the dental mesenchyme (Kettunen et al. 2000), and its receptors, Fgfr1b and Fgfr2b, are expressed in the epithelial cervical loops (Kettunen et al. 1998). Mesenchymal FGFs have been shown to enhance epithelial proliferation, and it is possible that the increase in epithelial proliferation in Tabby teeth may have caused the increase in cusp number. On the other hand, a link between FGFs and NF-κB signalling has been observed. In the chick limb the AER seems to be required for the expression of c-rel, an NF-κB subunit, in the progress zone. Exogenously applied FGF4 can substitute for this requirement (Bushdid et al. 1998; Kanegae et al. 1998). Furthermore, NF-κB is required for FGF mediated induction of Msx1 in the AER (Bushdid et al. 2001). As Edar also can activate NF-κB signalling (Yan et al. 2000; Koppinen et al. 2001; Kumar et al. 2001), it is not impossible that FGF10 may have contributed to the downstream effects of NF-κB signalling in Tabby teeth. FGF4 and FGF10 do not bind to the same receptors, but this nevertheless does not rule out the possibility of them regulating NF-κB signalling similarly.

In situ hybridisation analysis with markers for early bud- and cap-stage teeth showed that signal molecules associated with tooth development such as Fgf4, Wnt10a, L-fng, Fgf3, Fgf10, and Egfr were present in Tabby teeth. The size of the enamel knot was smaller correlating with the reduced number of cusps in adult teeth. This gives support to the hypothesis of the enamel knot being a signalling centre and determining tooth shape (Jernvall et al. 1994; Vaahtokari et al. 1996a).

### 4.3. Expression of ectodysplasin and Edar (I,II,III,IV, unpublished results)

Localisation of *ectodysplasin* and *Edar* mRNAs was studied with in situ hybridisation (see Table 3 for a summary of expression results). A combination of probes for ectodysplasin mRNA was used that recognised either all ectodysplasin splice forms (exon 1) or specifically Ta-A or Ta-B isoforms. The first results with probes for exon 1 or Ta-A 3’ terminus showed expression of *ectodysplasin* in the outer enamel epithelium in cap and bell stage tooth molars and in E14 epidermis prior to hair follicle formation. The expression patterns for exon 1 and Ta-A specific probes were identical suggesting that Ta-A splice forms are the major splice forms. Subsequently the detection for ectodysplasin mRNA was improved as a full-length cDNA probe was utilized. Comparison of *ectodysplasin* and *Edar* expression in embryonic tooth and skin revealed that *ectodysplasin* and *Edar* were both expressed in early oral and skin epithelium prior to placode formation (Laurikkala et al. 2001, 2002). Concomitant with placode formation *Edar* localised to the ectodermal placode but ectodysplasin expression continued in the intervening epithelium. Later *Edar* was seen in the dental signalling centres and in the hair bulb. Ectodysplasin continued to be expressed in adjacent epithelial tissues. The adjacent expression provoked the suggestion that ectodysplasin-Edar signalling mediates epithelial-epithelial communication (Laurikkala et al. 2001). Diffusion of ectodysplasin is likely to occur between these compartments as it is cleaved in the stalk region by furin and shed extracellularly (Chen et al. 2001; Elomaa et al. 2001; Schneider et al. 2001). This shedding is obligatory for the function of the protein as mutations in the furin cleavage site cause the ectodermal dysplasia phenotype in humans (Pääkkönen et al. 2001; Schneider et al. 2001; Vincent et al. 2001).
A comparison of *ectodysplasin* and *Edar* expression in tissues other than tooth and skin was conducted (Table 3). In the early mouse embryo *ectodysplasin-A* but not the *B* isoform was expressed in the visceral and definitive endoderm. No *Edar* was detected though (unpublished results). The developing brain showed a dynamic pattern of *ectodysplasin* and *Edar* expression. At some but not all locations the mRNA expression was complementary but never overlapping. *Ectodysplasin* localised to the lateral ventricles, thalamus, Rathke’s pouch, hindbrain, and to the ventral neuroepithelium along the mid-hindbrain border. It was also seen in some locations in the cranial mesenchyme. *Edar* was expressed in the diencephalon, lateral ventricles, ganglionic eminence, thalamus, and midbrain. Complementary expression patterns with *ectodysplasin* were seen in the thalamus, lateral ventricles, and midbrain. In the lacrimal glands *ectodysplasin* was expressed in the corneal epithelium and the *Edar* in the adjacent conjunctival epithelium and later in the lacrimal gland. In the submandibular salivary gland *ectodysplasin* was expressed in the surrounding mesenchymal tissue and *Edar* in the epithelial bud and branches.

*Edar* was also detected in the vibrissae epithelium, in the apical ectodermal ridge of the limb bud, and in the mammary placodes. *Ectodysplasin* was expressed in the anterior head mesenchyme but not immediately adjacent to the vibrissal *Edar* expression. In the limb and in the mammary epithelium *ectodysplasin* was not detected but this may reflect the low level of *ectodysplasin* expression rather than the absence of the transcript.

In summary, *ectodysplasin* and *Edar* mRNAs were found localised in adjacent epithelial compartments in the lacrimal gland, thalamus, midbrain, and lateral ventricles mimicking the expression pattern in teeth and hair. Teeth, hair, and lacrimal glands are all tissues that are affected in *Tabby* mice and HED patients. No brain defects are reported as primary symptoms although hyperthermia caused by inadequate sweating has been known to cause mental...
retardation in HED patients (Halperin and Curtis 1942; Clarke et al. 1987). Ectodysplasin has been reported to be expressed in the human brain during embryogenesis throughout the cerebral neuroepithelium. In the adult human brain Eda-positive neurons were found in the hypothalamus (Montonen et al. 1998). Secondly, ectodysplasin and Edar were expressed in adjacent epithelial and mesenchymal compartments in the developing submandibular gland at E13 and E14. The expression pattern suggests signalling between the epithelium and mesenchyme, and is the first indication of ectodysplasin-Edar signalling between epithelial and mesenchymal compartments. Ectodysplasin and Edar localisation has been studied in the submandibular gland using immunohistochemistry (Jaskoll et al. 2003). In these studies protein expression was not detected until E15. This may reflect either poor sensitivity of antibodies used or the fact that the proteins need not be translated until then. Ectodysplasin was localised to epithelial surfaces, which of course is necessary for signal transduction even if the mRNA is produced in the mesenchyme. Defective salivary glands are found in Tabby mice and HED patients (Blecher et al. 1983; Nordgarden et al. 2001). Thirdly, isolated expression of either the ligand or the receptor was seen in some tissues. For example, ectodysplasin was strongly expressed in the epithelial component of the developing pituitary gland, but no Edar was present. At least four different possibilities may account for these cases. First, expression of the mRNA need not result in expression of a functional and developmentally significant protein. Secondly, detection with in situ hybridisation may not be sensitive enough to detect low-level transcripts. Particularly ectodysplasin has been extremely difficult to detect. For example, Edar is expressed in the mystacial vibrissae but no localised ectodysplasin mRNA is seen in the vibrissae. Nevertheless, vibrissae of Tabby mice are defective to some degree suggesting that the ectodysplasin ligand must be present (Dun 1959). A third possibility is that ectodysplasin may signal via the Xedar receptor since ectodysplasin-A2 binds Xedar. Comprehensive Xedar expression patterns have not been reported and our attempts to localise Xedar mRNA in tissue sections have not been successful, leaving this possibility still open. The last alternative is that Edar binds a second ligand in addition to ectodysplasin. This has been suggested by the dissimilarity of the submandibular gland phenotypes of Tabby and downless mice (Jaskoll et al. 2003). Also it has been postulated that a phenotypic difference is seen in the enamel knot of Tabby and downless mice (Tucker et al. 2000). However, no such ligand has been found as yet.

4.4. Expression of TNFRSF19 (IV)

Given the large number of members of the TNFR superfamily, it is possible that other TNF signalling apart from ectodysplasin-Edar is also active during ectodermal organogenesis. Since the ectodysplasin and Edar null mutant phenotypes are fairly mild, it has been suggested that redundancy with other TNF receptors might account for the lack of a more severe phenotype (Mikkola and Thesleff 2003). TNFRSF19, also known as Troy or Taj, is an orphan TNF receptor (Hu et al. 1999a; Eby et al. 2000; Kojima et al. 2000). Its ligand-binding domain has a 50% identity with Xedar (Yan et al. 2000; Mikkola and Thesleff 2003), and a 33% homology to Edar (Kojima et al. 2000). It is expressed in hair follicles, and is thus a good candidate for functioning in a similar role as Edar. It should be kept in mind though that there are conflicting reports about its ability to activate NF-κB (Eby et al. 2000; Kojima et al. 2000).

The expression of TNFRSF19 was analysed during mouse embryogenesis and found to overlap with Edar in the tooth, vibrissae, limb bud, and mammary gland. In the tooth TNFRSF19 is expressed in the dental epithelium from E11 to E17. At the bud stage some expression is also seen in the dental mesenchyme. Edar is localised to signalling centres such
as the enamel knot during tooth morphogenesis but the expression of \textit{TNFRSF19} is more widespread. In the developing brain \textit{TNFRSF19} was expressed mainly in the dorsal fore- and midbrain in contrast to the more ventrally localised \textit{Edar} expression.

Thus, the expression pattern of \textit{TNFRSF19} suggests that it may mediate the same downstream signals as Edar in the tooth, vibrissae, limb bud, and mammary gland. The generation of \textit{TNFRSF19} mutant mice will assist in exploring the function of TNFRSF19.

4.5. \textit{Tabby} promoter cloning and its analysis in transgenic mice (I and unpublished results)

A genomic lambda clone (G2) of approximately 7 kb containing exon 1 of ectodysplasin was subcloned. The plasmid clones were sequenced and together with sequence obtained by PCR from G2 was assembled into a 3.5 kb contig. Analysis of the 2.5 kb preceding the transcription start site revealed a putative binding site for LEF1, which was conserved in the human \textit{Eda} promoter. Homology around the binding site was 96%, and throughout the promoter 82%. Due to the apparently low expression levels of \textit{ectodysplasin} mRNA a transgenic construct was generated where a 2.2 kb fragment of the promoter was used to direct beta-galactosidase expression. The aim was to further elucidate the \textit{ectodysplasin} expression pattern. Three founders (Ta-βgal ) were obtained which displayed nearly identical expression patterns. However, the pattern did not correspond to the endogenous pattern. For example, the teeth and hair did not show any staining. Instead, LacZ staining was seen at E10 in the somites (Figure 6a,b). The expression continued along the vertebra and ribs until at least E18 (data not shown). Cranial mesenchyme and lateral ventricles were also positive for the transgene, but the staining pattern was different from the endogenous \textit{ectodysplasin} expression in the developing head and brain (Figure 6c). This indicates that the 2.2 kb promoter fragment is not sufficient for reproducing endogenous \textit{ectodysplasin} expression. Comparison of the long intron I sequence (200-300 kb) in both mouse and human ectodysplasin genes may reveal enhancer elements required for transcriptional regulation (GenBank accession number NT_039711; Kere et al. 1996).

Figure 6. Tabby promoter betagalactosidase staining.

LacZ staining pattern of the Ta-βgal mice. (A,B) Transgene activity is seen in the anterior head mesenchyme and in the somites at E10. (C) Lateral ventricles (arrows) and the cranial mesenchyme are positive for beta-galactosidase at E12. (A,B) Whole mount LacZ staining. Scale bar for A, 1 mm, for B, 200 μm. (C) Frontal cryosection stained with Xgal. Scale bar, 500 μm.
4.6. Overexpression of Edar in transgenic mice (V and unpublished results)

Ectodysplasin can form trimers (II; Ezer et al. 1999; Schneider et al. 2001) like other members of the TNF ligand superfamily and when transfected to cells it activates the NF-κB pathway (Yan et al. 2000; Koppinen et al. 2001; Kumar et al. 2001). The genes activated by NF-κB and the downstream cellular effects are not understood. In an attempt to study Edar signalling transgenic mice were generated where the keratin 14 (K14) promoter directed expression of Edar in the basal epidermis, the outer root sheath of the hair follicle and the oral epithelium. In comparison to the endogenous expression transgenic Edar was seen first similarly in the developing epithelium albeit at higher levels, and thereafter the expression continued throughout the epithelium and was not confined to the forming placodes.

First, the phenotype of the transgenic mice depended heavily on the expression levels. The mice could be grouped into two main groups: strong and weak expression based on the transgene copy number and the protein expression levels. In addition, there were some intermediate types. Tissues affected by the misexpression included hair, teeth, nails, and mammary glands.

The main effect on the teeth was the change in cusp number and molar tooth shape. Surprisingly, different levels of Edar signalling in the oral epithelium had opposite effects on tooth morphology. Mice expressing Edar weakly had a reduced number of cusps, whereas strongly expressing mice an additional cusp. It is possible that the balance of promoters and inhibitors is very easily disturbed by subtle changes in Edar signalling. On the other hand, it cannot be ruled out that timing of the K14 promoter activity might be different in the weak and strong founder lines thus having a different effect on the enamel knot, which then results in different tooth shapes.

In addition, the tooth enamel was defective in the strong expression group. The phenotype was very similar to that observed with overexpression of ectodysplasin-A1 under the K14 promoter (Mustonen et al. 2003). Defective enamel can result from errors at different stages of amelogenesis and it is not clear what causes the enamel phenotype in Edar or ectodysplasin-A1 overexpressing mice. A curious detail is the similarity of the enamel defects with a transgenic mouse expressing the serine protease urokinase-type plasminogen activator in the oral and enamel epithelium (Zhou et al. 1999). It is therefore possible that Edar might regulate the proteolytic balance in amelogenesis. The fact that Edar signalling can have different effects in different tissues, e.g. lack of differentiated cells in the tooth, or promotion of ectopic organ formation (mammary gland, Mustonen et al. 2003), further stresses the importance of the cellular context for the downstream effects of TNF signalling.

The primary hair follicle (guard hair) defect was analysed in more detail. At E15 wild type mice have primary hair placodes. The strong expression K14-Edar mice had no primary hair follicles and the weak expression group had reduced numbers. This was reflected by the relative proportion of hair types in adult mice: the strong group had no guard hairs, and 1% of all hairs in the weak group were guard hairs. Control mice had 2% guard hairs. Later at E17 hair development commenced. However, in adult mice of the strong group the three remaining hair types did not differentiate properly as all the remaining hairs looked alike. Superficially they appeared to be awls, but they were shorter than awls and did not contain the characteristic air cell pattern typical for awls. Hairs of the tail were also shorter.
Molecular analysis showed the lack of placode markers at E15 epidermis. However, BMP4, which is normally expressed first throughout the mesenchyme and is then restricted to the placodal mesenchyme, remained expressed in the entire mesenchyme underlying the epithelium. This expression continued at least until newborn. Since Edar was expressed in the epithelium, BMP4 could not have been a direct target gene for Edar activated NF-κB. Nevertheless it is the first molecule shown to be upregulated as a consequence of Edar signalling. p21, which has been shown to be a target of BMP signalling (Jernvall et al. 1998), was also upregulated in K14-Edar mesenchyme.

Misexpression of Edar in the developing epithelium resulted in a loss of primary hair follicle formation. This could be caused by Edar activity in the interfollicular epithelium. The upregulation of BMP, which is a known inhibitor of hair follicle formation (Botchkarev et al. 1999), might inhibit placode formation. At present it has not been shown that Edar is truly active in the interfollicular area. However, the upregulation of Bmp4 throughout the mesenchyme suggests activity. In Tabby epidermis Bmp4 is downregulated at E15 even if the hair phenotype, lack of primary follicles, is similar (Laurikkala et al. 2002).

5. CONCLUDING REMARKS

The signalling pathways responsible for ectodermal organ developing are being at least partially unravelled at the moment. Members of the common growth factor families FGF, BMP, Shh, and Wnt seem to play major roles similarly as they play in the development of the early embryo and in the organogenesis of other organs as well. In C. elegans there is some evidence that organ-specific regulators exist (Gaudet and Mango 2002). The fact that mutations in five genes of the TNF signalling pathway turned out to be responsible for ectodermal dysplasias suggests that at least some members of the TNF family are specifically required for ectodermal organogenesis.

This work has focused on the ectodysplasin-Edar pathway in epithelial morphogenesis. This pathway is required for the development of teeth, hairs, sweat glands, salivary glands, lacrimal glands, and a large group of smaller glands, mainly in the nasal, oral, and pharyngeal area (Grüneberg 1971). In addition to vertebrates, Edar signalling is also required for ectodermal organogenesis in other species, as Edar mutations in Medaka fish result in lack of scale development (Kondo et al. 2001). It cannot be ruled out, however, that ectodysplasin-Edar signalling is utilised also in other organs. For example, the developing limb shows coexpression of Edar and TNFRSF19 in the AER. Ta/dl/cr mice have normally developed limbs, but it is possible that other TNF signalling, possibly mediated by TNFRSF19, compensate for Edar. NF-κB signalling is required for limb development in the chick (Bushdid et al. 1998; Kanegae et al. 1998).

What is known about the regulation of ectodysplasin-Edar signalling? First of all, several ectodysplasin mRNA splice forms have been detected, five of them in this study. Four splice forms have small changes in the TNF domain. This may affect the binding affinity as has been shown for ectodysplasin-A2. The A2 isoform seems to be coexpressed with the A1 isoform at later stages of hair follicle development (Yan et al. 2000). Differential regulation of the expression of the A3 and A4 isoforms has not been studied. The two shorter isoforms, ectodysplasin-B and –C, potentially could act as negative regulators by competing with the A1 or A2 isoforms. However, the lack of the TNF domain and the collagen-like Gly-X-X repeat capable of participating in multimere formation make this possibility somewhat
unlikely. Second, Wnt signalling mediated by LEF1 has been shown to regulate the expression of ectodysplasin (Laurikka et al. 2001, 2002; Durmowicz et al. 2002). This study showed that the promoter region containing the LEF1 binding site is not sufficient for mimicking endogenous expression. Since a similar phenotype was observed in three separate transgenic lines an integration effect can be ruled out. Therefore other enhancer sites and transcriptional regulators must contribute to proper expression of ectodysplasin mRNA.

How is Edar signalling mediated? Cloning of the crinkled gene and analysis of its protein product showed that the intracellular adapter protein for Edar is Edaradd (Figure 7) (Headon et al. 2001; Yan et al. 2002). It contains a DD domain that binds specifically to the DD domain of Edar. The similarity of the phenotypes of crinkled, downless, and Tabby mice further supports the specificity of Edaradd for Edar. Edaradd can bind TRAF1, -2, or -3 but not TRAF4, and only marginally TRAF5 or -6 (Headon et al. 2001; Yan et al. 2002). This is somewhat surprising since TRAF6 null mice have ectodermal dysplasia symptoms (Naito et al. 2002). However, Xedar does bind to TRAF6 (Naito et al. 2002; Sinha et al. 2002). One explanation of the TRAF6 null phenotype is that Xedar signalling is regulated by Edar. TRAFs 1-4 and –6 are all expressed in the mouse molar in an overlapping pattern so more detailed studies are required to resolve which TRAFs are used by Edar and Xedar (Ohazama et al. 2003). Edar can activate the NF-κB pathway (Yan et al. 2000; Koppinen et al. 2001; Kumar et al. 2001) but only slightly the JNK pathway (Kumar et al. 2001) indicating that NF-κB is the main signal transduction pathway of Edar. Further support for this comes from mice overexpressing IkB. This inhibition of NF-κB signalling resulted in an ectodermal dysplasia phenotype remarkably similar to Tabby mice (Schmidt-Ullrich et al. 2001). Moreover, IKKγ is required for Edar signalling (Döffinger et al. 2001). Human patients having hypomorphic mutations in IKKγ or lacking one copy of IKKγ display ectodermal dysplasia symptoms in addition to osteopetrosis and immune defects (Smahi et al. 2002; see also Table 1 for references). Complete lack of IKKγ in mice is lethal (Rudolph et al. 2000).

Figure 7. Schematic view of Edar and Xedar signalling.

Eda-A1 (ectodysplasin-A1) binds specifically to Edar, which recruits the adaptor protein, Edaradd. Edaradd can bind to TRAF1-3, and slightly to TRAF5 and -6. IKK complex activates NF-κB transcription factor. It is uncertain if Edar signalling can cause cellular death. Eda-A2 (ectodysplasin-A2) binds specifically to Xedar, which can directly recruit TRAF3 and -6. Both NF-κB and JNK pathways can then be activated.
What is the function of ectodysplasin-Edar signalling in ectodermal organs? What are the downstream targets that it regulates? Ectodysplasin has been overexpressed in transgenic mice using the K14 promoter (Mustonen et al. 2003). In contrast to overexpression of Edar, ectodysplasin is only active at sites where Edar is endogenously expressed, e.g. in hair and tooth placodes. In these mice ectopic organ formation is induced. Hair follicles are formed continuously, an extra molar is seen mesially to the first molar, and ectopic mammary glands are found along the mammary line. Furthermore, the amount of sweat is increased, and nails and hairs are longer than in wild type (Mustonen et al. 2003). Therefore Edar signalling can affect organogenesis at a relatively early stage, and in a fundamental way. In this work we have shown that Tabby molars are smaller than wild type molars and that the enamel knot is reduced in size (III). Could ectodysplasin affect proliferation? FGF10 can partially rescue the molar phenotype, and FGFs are known enhancers of proliferation. However, overexpression of ectodysplasin in the hair placodes does not significantly increase proliferation (Mustonen et al. 2003 and unpublished results) suggesting that proliferation may not be a primary response of Edar in hair placodes. In addition, Edar is localised to the enamel knot during tooth cap stage (Laurikkala et al. 2001). The enamel knot cells do not proliferate further arguing against proliferation as a direct effect of ectodysplasin-Edar signalling in the knot either. Apoptosis is a possible downstream effect of TNF signalling. Edar does not seem to induce apoptosis in cultured cells (Koppinen et al. 2001), although caspase-independent activation of the apoptotic pathway has been proposed by one study (Kumar et al. 2001). Tabby and downless tooth germs show comparable staining of apoptotic cells (Tucker et al. 2000; Koppinen et al. 2001). Inhibition of NF-κB signalling with a constitutively active IκBα in transgenic mice seems to increase apoptosis of hair follicles, suggesting that the role of NF-κB signalling in hair may be anti-apoptotic (Schmidt-Ullrich et al. 2001). It should be kept in mind though that as with all TNF signalling, the cellular context may effect Edar signalling, and that the downstream responses possibly differ at different stages of organogenesis.

We have also shown that BMP4 is upregulated in K14-Edar dermis (V). There are at least two ways of explaining this. First, BMP4 may be an indirect target of Edar signalling. Edar in the epithelium activates NF-κB, which then transcriptionally activates an unknown factor, which directly or via mediators induces BMP4 expression in the dermis. The other possibility is that BMP4 expression, seen throughout the dermis prior to placode formation, remains in a default status when primary hair placodes do not form in the transgenic skin. However, the fact that BMP4 is missing in Tabby skin of similar age, which also lacks hair placodes, argues against this interpretation (Laurikkala et al. 2002). MAdCAM-1, an adhesion molecule, has been proposed as a target of Edar signalling since MAdCAM-1 is expressed in hair placodes and this expression is missing in Tabby mice (Nishioka et al. 2002). It also has an NF-κB binding site in its promoter. No direct evidence to support this exists though.

In summary, since cloning of ectodysplasin in 1997 much has been learned about its signalling pathway. Nevertheless, we are forced to return to the original question: what is the fundamental function of ectodysplasin in ectodermal organogenesis? It is the task for the next few years to put together the missing jigsaw puzzle pieces. A combination of novel techniques such as global analysis of gene expression techniques (Cui et al. 2002) and older cell biology assays will probably be needed to answer these questions.
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Johanna Pispa
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