Canonical Wnt Signaling in Hair and Mammary Gland Patterning and Development

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A 14-day old mouse embryo showing green fluorescent protein expression under control of keratin17 promoter at sites of ectodermal organ development.

“ I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale. “

Marie Curie

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original articles (referred in the text by their Roman numerals) and unpublished results.

I Närhi, K., Järvinen, E., Birchmeirer, W., Taketo, M.M., Mikkola, M.L.*, and Thesleff, I*. (2008) Sustained epithelial beta-catenin activity induces precocious hair development but disrupts hair follicle down-growth and hair shaft formation. *Development* 135(6): 1019-28.

II Närhi, K., Tummers, M., Ahtiainen, L., Nobuyuki, I., Thesleff, I., and Mikkola, M.L. (2012) Sostdc1 regulates the size and number skin appendages. *Developmental Biology* 364(2): 149-161.

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ABBREVIATIONS

ActR	Activin receptor
AER	Apical ectodermal ridge
APC	Adenomatosis polyposis coli
APCCD1	Adenomatosis polyposis coli downregulated 1
B.C.	Before Current Era
BMP	Bone morphogenetic protein
BMPR	Bone morphogenetic protein receptor
BrdU	Bromodeoxyuridine, synthetic nucleoside
CELSR1	Cadherin, EGF LAG seven-pass G-type receptor 1
CKI	Casein kinase I
CTGF	Connective tissue growth factor
CTHRC1	Collagen triple helix repeat-containing protein 1
DKK	Dickkopf
DVL	Dishevelled
E	Embryonic day
EDA	Ectodysplasin
EDAR	Ectodysplasin receptor
EDARADD	Edar-associated death domain
EGF	Epidermal growth factor
ERBB	Vertebrate homolog of avian v-erb-b2 erythroblastic leukemia viral oncogene, neuro/glioblastoma derived oncogene
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FZ	Frizzled Wnt-receptor
GDF	Growth and differentiation factor
GFP	Green fluorescent protein
GLI	Glioma-associated oncogene hedgehog
GSK3β	Glycogen synthase kinase 3 beta
HED	Hypohidrotic ectodermal dysplasia
HH	Hedgehog
HIP	Hedgehog interacting protein
HR	Hairless
HSPG	Heparin sulfate proteoglycan
ID	Inhibitor of DNA binding
IG	Immunoglobulin domain
I-κB	Inhibitor of kappa B
IRS	Inner rooth sheath
JNK	c-Jun N-terminal kinase
K	Keratin
LEF1/TCF	Lymphoid enhancer factor1/T-cell factor
LGR	Leucine-rich repeat-containing G protein-coupled receptor

LRP	Low density lipoprotein receptor-related protein
MAP	Mitogen-activated protein
MMTV	Mouse mammary tumor virus
mRNA	Messenger ribonucleid acid
MSX	vertebrate homolog of Drosophila muscle segment (Msh) homeobox gene
NF-κB	Nuclear factor κB
NRG	Neuregulin
ORS	Outer root sheath
P	Postnatal day
p21	Cyclin-dependent kinase inhibitor
PCP	Planar cell polarity
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PFA	Paraformaldehyde
PTC1	Patched1
PTHR	Parathyroid hormone receptor
PTHrP	Parathyroid hormone-related protein
RANKL	Receptor activator of Nf-κb-ligand
ROR2	Receptor tyrosine kinase-like orphan receptor 2
R-Spondin	Reelin domain-containing spondin
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcriptase polymerase chain reaction
RYK	Receptor-like tyrosine kinase
SEF	Similar expression to Fgf genes
SKA	Scaramanga
SOX2	SRY-box containing gene 2
SRC	Rous sarcoma oncogene
sFRP	Soluble Frizzled-related protein
SHH	Sonic hedgehog
SMAD	Vertebrate homolog of Drosophila mothers against decapentaplegic (Mad) gene
SMURF	Smad ubiquitination regulatory factor-1
SOS	Vertebrate homolog of Drosophila son of sevenless gene
SOST	Sclerostin
SOSTDC1	Sclerostin domain containing 1
^{35}S-UTP	Uridine 5'[α-35S]thiotriphosphate
TBX	T-box
TCF	T-cell factor
TEB	Terminal end bud
TGF-β	Transforming growth factor beta
TNF	Tumor necrosis factor
TRAF	Tnf receptor-associated factor
USAG-1	uterine sensitization-associated gene-1
WIF	Wnt inhibitory factor
WNT	Vertebrate homolog of the Drosophila Wingless gene

ABSTRACT

Although mammary glands and hair are morphologically and functionally different organs, they share similar early developmental features and arise from ectoderm like other skin appendages. Their development begins by the formation of an epithelial placode and a mesenchymal dermal condensate and crosstalk between these tissue compartments directs the subsequent developmental steps resulting in epithelial morphogenesis and the generation of specific organ shapes. Different types of hair filaments are observed in various anatomical regions and are produced by hair follicles consisting of several epithelial cell layers and a dermal papilla. The mammary gland is constructed of a nipple rising above the skin and the glandular mammary tree producing milk. Both organs continue development postnatally; new hair is produced by repeated hair cycles lasting throughout the lifetime and postpubertal mammary ductal tree is remodelled upon pregnancy and following lactation and involution. Handful of conserved signaling pathways guides both the embryonic and postnatal developmental steps of skin appendages. Hair and mammary gland development are especially known to depend on signals from the β-catenin-mediated Wnt pathway. The Wnt pathway is highly complex with multiple ligands, receptors, and signaling modulators, and cross-talk with other signaling pathways is apparent. Here, I have examined the role of Wnt signaling in hair and mammary gland patterning and development, and also analysed the interactions and hierarchical order of Wnt pathway with other signaling molecules in this context. The study has involved three different mouse models in which Wnt signaling is modulated either by continuous activation of β-catenin, inactivation of the Wnt and Bmp pathway regulator Sostdc1, or ablation of a Wnt target gene, *Fgf20*. Continuous Wnt/β-catenin signaling in embryonic ectoderm in $Catnb^{\Delta ex3K14/+}$ mice caused precocious hair development and, the formation of ectopic and mispatterned hair placodes showing disturbed morphogenesis and hair filament formation. *Fgf20*-null mice showed a surprisingly early hair phenotype with a loss of expression of several dermal condensate markers but presence of grossly normal morphological patterning of placodes with altered placode marker expression patterns. Loss of Sostdc1 had very mild effects on pelage hair development but interestingly, Sostdc1 appears to play a role in determing correct vibrissal hair and nipple number and the regulation of mammary bud size/form, plausibly through inhibitingWnt signaling.

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Helsinki, May 2012

Katja Närhi

1 REVIEW OF THE LITERATURE

1.1 Skin appendage development

The integument consisting of the skin and its appendages provides barrier between body and the environment. By gaining variations or novelties in molecular mechanisms, the integument has evolved and allowed animals to adapt to various ecological environments. Different animals species show a variety of skin appendages like hairs, feathers, teeth, horns, scales, nails, and different exocrine glandular organs like mammary and sweat glands which all derive from ectoderm. Although the eventual shape of these organs and the function varies greatly, they share common embryonic developmental processes and molecular pathways that guide morphogenesis and patterning. Skin appendage development involves epithelial-mesenchymal interactions (Hardy, 1992; Kollar 1970; Sengel, 1976). The mesenchymal cells originate from different parts of the body; neural crest cells serve as progenitors for tooth and vibrissal hair mesenchyme whereas mesodermal cells give rise to the dermal cells of trunk hairs and mammary glands. Classical tissue recombination studies between species or different body regions have suggested that mesenchyme harbors the instructive signals for correct organ shape (Dhouailly, 1973; Kratochwil, 1969; Kusakabe et al. 1985; Sengel, 1976).

The first morphological feature of development is the appearance of a local epithelial thickening, called placode, which invaginates to the underlying mesenchyme to form a bud. Mesenchymal fibroblasts respond to placodal signals by forming a dermal condensate under the placodes and buds. In tooth, hair, and feather development this mesenchymal condensate is later enveloped by the folding epithelium to form a dermal papilla which does not appear in the glandular organs. (Pispa and Thesleff, 2003; Mikkola, 2007). The epithelial bud of every organ folds or branches in an organ-specific manner to form the proper adult structure and morphogenesis often continues postnatally, with some of the organs like nails growing throughout the adult life. (Pispa and Thesleff, 2003; Mikkola, 2007).

The development of skin appendages relies on inductive tissue interactions mediated by a set of conserved signaling molecules which belong to a limited number of families. These include the Wnt, fibroblast growth factor (Fgf), transforming growth factor beta (Tgfβ), ectodysplasin (Eda), hedgehog (Hh) and Notch families. The regulatory molecules provide either promoting or inhibitory signals, which are reciprocally sent between the epithelium and mesenchyme to govern skin appendage morphogenesis. The hierarchy of these signals varies with context. However, the contributing cellular mechanisms, like proliferation or migration, governing the initiation of skin appendage formation are not well understood. (Pispa and Thesleff, 2003).

1.2 Conserved signaling pathways guiding organ development

"Signaling pathways are an ever present force in every animal's life"
–Gordon & Nusse, 2006

Aristotle (384-322 B.C.), interested in organ formation, was carrying out comparative embryology studies (Peck, 1968) and approximately a century ago genes were thought govern the phenotype. Over a decade ago, however, developmental biologists have truly begun to understand how organs form and especially to realize the complex molecular mechanisms behind developmental processes.

From insects to mammals, there are only a few cell-cell signaling networks forming the basis for the regulation of both embryonic organ development and adult tissue homeostasis and regeneration. Furthermore, it appears that the mechanism for each pathway has been conserved during evolution. Usually gene duplications during evolution have increased the number of similar signaling family members, which may offer redundant functions and provide insensitiviness to the effects of mutations that would otherwise disturb developmental processes. However, these duplications may also serve specific functions, thus increasing the complexity of signaling.

How is the variety of organs built by a relatively small number of tools? Nature has created more complex regulatory networks by allowing signaling pathways to interact with each other in space and time. Furthermore, the activity of each signaling pathway is controlled at several levels e.g. at the transcriptional or protein level, or extracellularly by molecular regulators affecting ligand binding to receptors. The use of paracrine inductive molecules, morphogens, which diffuse various distances, has created more flexibility to the regulatory processes, as well. Morphogens are able to determine formation of specific cell types according to different morphogen concentrations sensed by the responding cell (Ashe and Briscoe, 2006).

Although the key genes involved in the major signaling pathways are largely known it is still unclear how in a specific network the components function together to regulate any given developmental stage during organ formation. In the following chapters, I will present selected important signaling pathways and molecular regulators important for skin appendage development in vertebrates.

1.2.1 Wnt

Integrase-1 (Int), the vertebrate homologue for the *Drosophila* segment polarity gene *Wingless,* identified in 1982 (Nusse and Varmus, 1982) and later renamed as Wnt1, is the founding member of the large Wnt family (Rijsewijk et al. 1987). Human and mouse Wnt proteins include 19 secreted and lipid-modified glycoproteins with a conserved pattern of 22-24 cysteine residues The complex mechanisms of Wnt signaling are highly conserved and the Wnt signals play a central role in embryonic development and in adult homeostasis. Wnt signals regulate tissue patterning, cell polarity, proliferation, directed migration, and determine cell fates. (vanAmerongen and Nusse, 2009). Moreover, they have been shown to expand and maintain stem cells and are involved in tissue regeneration. Deregulation of pathway activity may lead to cancer and several degenerative diseases (Logan and Nusse,

2004; Klaus and Birchmeier, 2008; Rey and Ellies, 2010).

Wnt signaling involves the transduction of signals through several pathways. The complexity of Wnt signaling is further characterized by different receptors or receptor complexes for several Wnt ligands and a great number of transcription factors. (Kikuchi et al. 2009; VanAmerongen and Nusse, 2009). Moreover, as recent studies have suggested it is worth to noting that instead of linear Wnt pathways, the Wnt ligands mediate signals through different pathways depending on cellular context (VanAmerongen and Nusse, 2009). Thus, Wnt proteins form a complex regulatory pathway, regulated at several levels by negative feedback loops and cross-talk with other pathways, causing multiple outcomes. Classically, the Wnt pathways are divided into the β-catenin-dependent canonical, and the β–catenin-independent non-canonical pathways. But as reviewed by VanAmerongen and Nusse (2009) it is becoming obsolete to strictly divide the Wnt ligands and the different receptor types themselves into classes with specific activities.

1.2.1.1 β-catenin-dependent Wnt pathway

β-catenin is the central component of the intensively studied canonical pathway thought to be involved in several processes such as pattern formation and osteogenesis and the cause for cancer (Clevers, 2006). Loss of β-*catenin* leads to early lethality in mice (Huelsken et al. 2001) and analysis of conditional β-*catenin* loss- and gain-of function mice have shown that this Wnt pathway component affects development of various organs, like a number of internal organs, sensory organs, skin appendages, bone, limbs, and central nervous system (Grigoryan et al. 2008)

The pathway mechanism of canonical signaling is represented in Figure 1. Wnt ligands are released from the cell with help from the multi-pass transmembrane protein Wntless and they are thought to remain attached to cell membrane or extracellular matrix due to their hydrophobic nature. Wnts recognize their specific receptor in a context-dependent manner. Ligand introduction to its receptor appears to be mediated by heparin sulfate proteoglycans (HSPGs), which possibly also serve as mechanism in the transport of Wnt ligands between cells. Wnt may exert its functions 20-30 cell diameters away from the producing cell. Thus, these proteins are able to exert both short and long range signaling. The canonical pathway involves several Frizzled (Fz) receptor family members consisting of seven-pass transmembrane proteins which carry an extracellular cysteine-rich domain required for binding Wnts. To transduce signals, Fz acts together with the single-pass transmembrane co-receptors of the low-density lipoprotein receptor-related protein (Lrp) family forming a ternary complex with Wnts . Out of the 12 members, Lrp5 and Lrp6 are thought to mediate signals of the canonical pathway (Gordon and Nusse, 2006; Rey and Ellies, 2010; van Amerongen and Nusse, 2009). Lrps may also negatively regulate Wnt activity, as has been shown for Lrp1 and Lrp4 (Zilberberg et al. 2004; Ohazama et al. 2008). In addition, leucine-rich repeat containing G protein-coupled receptors (Lgr) 4, 5 and 6, which bind one of the four reelin domain-containing spondin (R-spondin) ligands, have been shown to associate whith the Fz-Lrp receptor complex to enhance canonical Wnt signaling (Carmon et al. 2011; De Lau et al. 2011).

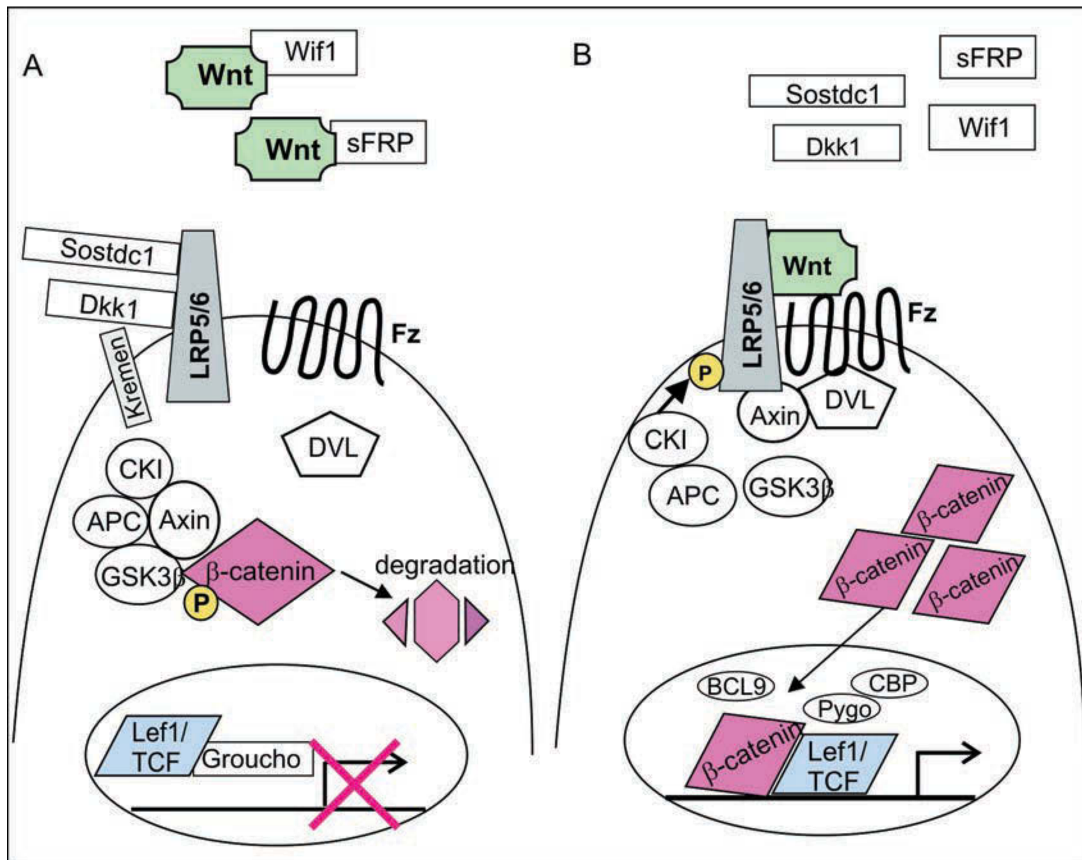

Figure 1. β-catenin dependent (canonical) Wnt pathway

(A) Wnt signaling is kept quiescent by extracellular antagonists, suchs as sFrp and Wif1, which bind Wnt ligands, or Sostdc1 and Dkk1, which bind single-pass transmembrane co-receptors Lrp5 or 6. Kremen augments Dkk mediated negative regulation. Cytoplasmic β-catenin is phosphorylated by an inhibitory complex (Apc-Gsk3β-Axin) and degraded by proteolysis. Nuclear Lef1/Tcf forms a complex with the repressor Groucho to block expression of Wnt target genes.
(B) A free Wnt ligand binds to the seven-pass transmembrane receptor Fz and they form a complex with Lrp5/6 leading to activation of the intracellular Dvl, which presumably binds to Fz. The cytoplasmic tail of Lrp5/6 is phosphorylated by Cki and this allows interaction of Axin and Lrp, thus leading to inactivation of the inhibitory complex. Cytoplasmic β-catenin accumulates and travels to the nucleus, where it binds to Lef1/Tcf replacing the bound repressors and the formed complex induces target gene expression together with other transcription factors, like Pygo, Cbp [CREB (cAMP response element-binding) binding protein], and Bcl9 (B cell CLL/lymphoma 9). (Gordon and Nusse, 2006).

In the absence of Wnt ligand, the destruction complex consisting of Axin, Adenomatosis polyposis coli (Apc), Glycogen synthase kinase 3β (Gsk3β), and Casein kinase I (Cki) captures and phosphoporylates cytoplasmic β-catenin. Phosphorylated β-catenin is targeted to destruction by a proteosome. Activated Fz/Lrp receptors cause inhibition of the destruction complex through actions of the cytosolic phosphoprotein Dishevelled (Dvl)

although the mechanism of its function is unclear. Axin has been shown to be involved in the destabilization as well, by binding to the cytoplasmic tail of Lrp. Free β-catenin accumulates in cytoplasm and translocates to the nucleus to interact with the lymphoid enhancer factor/ T-cell factor (Lef1/Tcf)) family of transcription factors and with other transcription factors in cell-type specific manner to regulate gene expression. (vanAmerongen and Nusse, 2009).

In addition to acting as a trancriptional activator, β-catenin has also been found to be a structural protein in cell-cell adherens junctions (Barth et al. 1997; Ben-Ze'ev and Geiger, 1998). It has been demonstrated, that most of β-catenin is bound to E- and P-cadherins and α-catenin forms a bridge to the actin cytoskeleton only when a small number of cytoplasmic β-catenin is stabilized in response to Wnt signaling (Adams and Nelson, 1998; Heasman et al. 1994 ; Yap et al. 1997).

The canonical pathway is regulated by several modulators at virtually all levels depending on the cellular context. Extracellular soluble Dickkopf (Dkk) family member, Dkk1, binds to Lrp5/6 to cause the internalization of the receptors. A single-pass transmembrane receptor, Kremen augments this negative regulation exerted by Dkk but when Dkk is not present, Kremen has been shown to perform stimulatory activities instead. (VanAmerongen and Nusse, 2009). Several secreted and soluble cysteine-knot containing proteins have also been suggested to regulate Wnt signaling by binding to Lrps (Rey and Ellies, 2010). These include Sclerostin (Sost), Sclerostin domain containing 1 (Sostdc1; discussed in more detail below), and Connective tissue growth factor (Ctgf) (Ellies et al. 2006; Itasaki et al. 2003; Mercurio et al. 2004). Other extracellular soluble antagonists are Wnt inhibitory factor (Wif) and soluble Fz related proteins (sFrp) which inhibit and bind Wnts directly. Furthermore, collagen triple helix repeat-containing protein 1 (Cthrc1) is able to bind to several Wnt ligands, Fzs and receptor tyrosine kinase-like orphan receptor 2 (Ror2) mainly involved in the non-canonical Wnt signaling. Studies imply that Cthrc1 stimulates Wnt-Fz-Ror2 complex formation at the expense of Wnt-Fz-Lrp6 complexes. (vanAmerongen and Nusse, 2009). In the nucleus, Nemo-like kinase negatively regulates Tcf by phosphorylation, and Inhibitor of catenin and Chibby antagonize β-catenin activity (Ishitani et al. 2003; Rey and Ellies, 2010).

1.2.1.2 β-catenin-independent Wnt pathways

To date most of the work in the Wnt field has concentrated on β-catenin-dependent Wnt signaling but examples continue to accumulate in which Wnts and/or other key components of the canonical signaling cascade participate in β-catenin-independent processes. The several non-canonical Wnt pathways involving planar cell polarity (PCP), Ca^{2+}, and Receptor-like tyrosine kinase (Ryk), have been shown to regulate adipogenesis, calcium homeostasis, and apoptosis to mention a few. (Rey and Ellies, 2010; Sugimura and Li, 2010).

PCP mediates signals without recruiting β-catenin/Tcf complexes to regulate cell polarity and polarized cell migration (Simons and Mlodzik, 2008;Wansleeben and Meijlink, 2011). The signals are transduced through Fz receptors and Dvl similar to the canonical pathway but different downstream components are included like small Rho GTPases (small G-proteins), Rac, Dishevelled associated activator of morphogenesis, and c-Jun N-terminal kinase (Jnk). The prevailing question has been which molecule activates Fz for PCP signaling? It appears, that flies activate PCP independently of Wnt but vertebrates require Wnt for this

signal transduction. (Rey and Ellies, 2010; VanAmerongen and Nusse, 2009).

Fz receptors are also able to activate the Ca^{2+} pathway to release intracellular calcium to regulate cell adhesion and movements during gastrulation. Here, Wnts bind to Fz and Ror receptors, which are atypical receptor tyrosine kinase (RTK) family members, and the downstream components likely involve Nuclear factor of activated T cells, phospholipase C, and phosphokinase C. Otherwise the genes activated by the pathway are still unknown. (Kohn and Moon, 2005; VanAmerongen and Nusse, 2009).

During the development of the central nervous system, Wnt protein gradients guide the direction of extending axons by signaling through a Ryk receptor, an atypical member of the RTK family with a single-pass transmembrane domain, leading to the activation of Rous sarcoma oncogene (Src) proteins (Liu et al. 2005; Schmitt et al. 2006; Yoshikawa et al. 2003). A number of Wnts, including Wnt5a have been shown to act through Ryk as axon repellents. (Liu et al. 2005).

1.2.1.3 Crosstalk between Wnt and other signaling pathways

Although each signaling pathway is capable of functioning independently as they have their own ligands, receptors, and nuclear signal transducers, they may also cross-talk to guide different biological events. Wnt signaling has been suggested to interact with several of the other classical signal transducing pathways causing synergistic or antagonistic effects depending on cellular context. The cross-talk may occur at the extracellular, intracellular, or nuclear level. It has been suggested that the interactive nature of the Wnt pathway could be partly due to its requirement to stabilize transcriptional events caused by other mechanisms and to avoid unwanted transcriptional activities (Arias and Hayward, 2006). Below a few examples of interactions are represented mainly focusing on cross talk with Tgfβ/Bone morphogenetic protein (Bmp) and Fgf signaling.

Wnt and Bmp pathways often regulate similar biological processes such as organogenesis, stem cell maintenance, and carcinogenesis. Over a decade ago it was reported that during tooth development Bmp4 activates *Lef1* expression, which is also a target gene of Wnt signaling (Kratochwil et al. 1996; Filali et al. 2002). More recently, Smad4, the intracellular downstream component of other Tgf-β signaling, was shown to form a complex with β-catenin or Lef1 to synergistically regulate gene expression (Nishita et al. 2000; Nawshad and Hay, 2003; Lim and Hoffman 2006; Nawshad et al. 2007). This kind of interaction could also lead to competition between the pathways for the available Smad4 components. Furthermore, Bmp and Wnt pathways appear to share also some extracellular modulators, mostly the cysteine-knot proteins like Ctgf, Sost, and Sostdc1, which are able to bind to Lrp5/6 receptors (Itasaki et al. 2003; Kusu et al. 2003; Laurikkala et al. 2003; Mercurio et al. 2004; Rey and Ellies, 2010). These modulators may simultaneously modulate Bmp and Wnt pathway activities by using different structural domains when binding their target molecules.

A growing number of studies have reported intricate interactive events in *Drosophila* but also in vertabrates (Itasaki and Hoppler, 2010) for example during limb development and bone formation (Soshnikova et al. 2005; Johnson et al. 2004; Katagiri et al. 2008). It has been suggested that in limb primordia during apical ectodermal ridge (AER) formation, Bmp receptor 1a (Bmpr1a) signaling, which may induce *Fz1,* is required upstream of β-catenin as

β-catenin signaling could rescue the deficits in AER formation caused by ablation of *BmprIa* (Soshnikova et al. 2005). Further, it was shown that β-catenin could activate *Bmp4* thereby creating a positive feeback loop that amplifies Bmp activity. The specification of the dorsal-ventral axis of the forming limb, however, was detected to be dependent on a parallel or reversed hierarchical order of Bmpr1a and β-catenin signaling. (Soshnikova et al. 2005). During bone formation, osteoblast differentiation appears to require negative regulation of Wnt activity by Bmp but in the differentiated cells both signaling pathways function synergistically (Katagiri et al. 2008).

It has been proposed that Bmp and Wnt pathways interact in four fundamentally different ways. In mutual regulation, both pathways regulate each other's expression and extracellular crosstalk involves extracellular molecules which bind to ligands or receptors of both pathways causing negative or positive regulation. Intracellular crosstalk interferes with or enhances one pathway by signaling components of the other pathway. Combinatorial transcriptional regulation occurs when the signal transduction mechanisms of both pathways are integrated in a co-operative or antagonistic way by means of *cis* regulatory enhancer and promoter sequences to regulate expression of target genes. (Itasaki and Hoppler, 2010).

In recent years, an increasing number of examples of interactive signaling between Fgf and Wnt have been reported, often showing synergistic effects (Eblaghie et al. 2004; Keenan et al. 2006; McGrewa et al. 1997; Shimogori et al. 2004). It has been reported that during bone and tooth development, the canonical Wnt pathway activates directly Fgf18 and Fgf4, respectively (Kratochwil et al. 2002; Reinhold and Naski, 2007) but studies in *Xenopus* have proposed that also Fgf signaling may enhance Wnt activity by negatively regulating the repressor function of the nuclear factor Groucho which is known to inhibit Lef1/Tcf activity (Burks et al. 2009). More intricate crosstalk was shown in cell migration studies with zebrafish. Wnt/β-catenin and Fgf signaling pathways maintain the polarity of the zebrafish lateral line primordium during migration through interactions of the pathways that serve to restrict activation of both pathways. Fgf was suggested to inhibit Wnt signaling through inducing Dkk1, whereas Wnt, induced the expression of a Fgf inhibitor, similar expression to Fgf genes (Sef). (Aman and Piotrowski, 2008). Recently it was suggested that during salivary gland branching, lumen formation requires Fgf signaling to inhibit Wnt activity by inducing the Fgf target gene, *sFRP,* whose protein product serves to antagonize Wnt signaling (Patel et al. 2011).

1.2.2 Hh

Hh signaling is required for normal development of basically all organs and in some contexts the inhibition of signaling activity seems to play an equally important role as the active pathway. Hh protein was originally discovered in *Drosophila* (Nüsslein-Volhard and Wieschaus, 1980) and the three vertebrate counterparts for it are Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh). Ihh regulates bone morphogenesis and Dhh is involved in testis development (Lanske et al. 1996; Bitgood et al. 1996) but Shh appears to exert the greatest number of functions. Hedgehog pathway activity regulates pattern formation, promotes proliferation, determines cell types, and thus, creates tissue boundaries. Further, it is crucial for limb and neural differentiation, required in left-right asymmetry regulation, and involved in stem cell maintenance in adult tissues including the brain and epithelia of internal organs.

(Ingham et al. 2011). Misregulation of the pathway can cause dramatic developmental defects in humans like Gorlin syndrome, Greig cephalopolysyndactyly syndrome, or cancer, especially the skin-derived common basal cell carcinoma. (Jiang and Hui, 2008; McMahon et al. 2003; Niewenhuis and Hui, 2005).

Genetic and biochemical studies in *Drosophila* have largely contributed to the current knowledge of the hedgehog pathway mechanism in mammals (**I**ngham et al. 2011). The signaling network involves two cell types usually located in different tissue compartments like epithelium and mesenchyme. To become functional paracrine factors, hedgehog ligands need to be catalytically cleaved by cholesterol and further lipid-modified by cholesterol and palmitate to allow the diffusion of hedgehogs. Hh ligands can have either short-range effects if it is tethered to the plasma membrane of the signaling cell or long-range effects as a diffusible ligand when released from the signaling cell. HSPGs have been suggested to promote the long-range diffusion of Hh.

Hh binds to its transmembrane receptor Patched1 (Ptc1), which undergoes conformational change and releases the inhibitory effect on the other transmembrane protein, the obligatory signal transducer Smoothened (Smo). Ptc1 may also function as a ligand sequesterer and thus, restricts the range over which Hhs signal. Furthermore, ligand binding to Ptc1 can be inhibited by the competing actions of Hedgehog interacting protein (Hip), or glypican family member 3 expressed by the responding cell. (Ingham et al. 2011). *Hip1* is a Hh primary target gene and thus, forms a negative feedback loop to restrict Hh signaling (Jeong and McMahon, 2005). Other direct targets for Shh are thought to be *Ptc1* and *Glioma-associated oncogene hedgehog 1* (*Gli1*) (Jia and Jiang, 2006). Hh signaling has been shown to rely on primary cilia, which are small cellular projections present in a number of vertebrate cells. Intraflagellar transport, a process needed in the assembly and maintenance of cilia, is crucial in trafficking Hh pathway components like Smo and Glis through the cilium (Goetz et al. 2009; Ingham et al. 2011).

The three vertebrate Gli transcription factors Gli1, Gli2, and Gli3, are located cytoplasmically and attached to microtubules in the absence of Hh. Furthermore, they are proteolytically cleaved by kinases to produce a repressor form of the Gli. Upon Hh binding to Ptc1, activated Smo releases Glis from microtubules and prevents the proteolysis of these transcription factors, thus, promoting the formation of Gli activator which is translocated to the nucleus to regulate gene expression (Ingham et al. 2011). Gli1 as a transcriptional target of Hh signaling is involved in positive feedback signaling to reinforce the Hh pathway activity. According to genetic and biochemical studies Gli2 and Gli3 are the primary mediators of Hh signalling. In general, Gli2 functions as an activator and Gli3 as a repressor but in some developmental contexts Gli2 may repress and Gli3 activate Hh signaling (Jia and Jiang, 2006). In addition, analyses of compound mouse mutants have proposed that Gli genes have genetic interactions (Ingham and McMahon, 2001).

1.2.3 Eda

Ectodysplasin (Eda) and its receptor Edar belong to the tumor necrosis factor (Tnf) and Tnf receptor superfamily which plays a central role in immune responses but also regulates tissue remodelling, as well (Ferguson et al. 1997; Mikkola et al. 1999; Monreal et al. 1998; Srivastava et al. 1997). Tnf signaling promotes cell survival through activation of Nf-κb (nuclear factor

kB) or Jnk or can lead to caspase-dependent cell death (Baker and Reddy, 1998).

Several alternatively spliced human or murine transcripts of *Eda* are formed of which the two longest and highly conserved *Eda-A1* and *Eda-A2* are known to produce functional ligands. Eda-A1 and Eda-A2 have similar protein structure showing a short intracellular N-terminus and a single transmembrane domain followed by an extracellular collagenous domain and the conserved C-terminal Tnf homology domain. The ligands differ only by insertion of two amino acids in the C-terminus. Another difference is in their receptor binding specificity: Eda-A1 binds to type I transmembrane receptor Edar and Eda-A2 recognizes type III transmembrane protein Xedar. (Mikkola and Thesleff, 2003). Troy has been indentified as a third receptor type (Kojima et al. 2000), closely relating to Edar and Xedar, but the ligand and signal transduction mechanism are still unknown. Typical to Tnf ligands, Eda is produced as a type II transmembrane protein. (Mikkola et al. 1999). To become biologically active, the extracellular part of Eda is cleaved from the cell surface by calcium-dependent serine endoprotease furin. *In vitro* studies have suggested that similar to other Tnf signalling, the effects of Eda/Edar signaling are mediated through activation of Nf-κb, but it is possible that the Edar pathway slightly activates the Jnk pathway, as well. Furthermore, studies have shown that Xedar signaling may also stimulate Nf-κb via the Jnk pathway, and induce caspase-dependent apoptosis. The canonical Nf-κb pathway used by Eda-A1 involves ligand-activated Edar which through its death domain binds to an adaptor protein Edar-associated death domain (Edaradd) which mediates the signal to Tnf receptor-associated factors (Traf) to activate the cytoplasmic dimeric transcription factor Nf-κb. The nuclear targeting signal of Nf-κb is masked by inhibitor of kappa B (I-κB) which is sent to proteosome-mediated degradation after its phosphorylation by Traf-induced I-κB kinase complex. Thus, Nf-κb is free to travel to nucleus to regulate gene transcription. (Mikkola and Thesleff, 2003).

Inactivating mutations in human *Eda*, *Edar*, or *Edaradd* cause hypohidrotic ectodermal dysplasia (HED) of identical phenotype (Kere and Elomaa, 2002). The most common form is the X-linked HED caused by inactivated *Eda* (Kere et al. 1996; Pääkkönen et al. 2001). HED patients show defects in several ectodermal organs including the absence of sweat glands, abnormal teeth and nipples, sparse hair, and defective skin glands (Reed et al. 1970; Clarke et al. 1987; Pinheiro and Freire-Maia, 1994; Kere et al. 1996). Analogous mouse models for human HED include *tabby* caused by spontaneous mutations in *Eda* (Falconer, 1952; Srivastava et al. 1997), *downless* and *Sleek* with mutated *Edar* (Headon and Overbeek, 1999), and *crinkled* mutants with inactivated *Edaradd* (Headon et al. 2001; Yan et al. 2002). Furthermore, mice with suppressed Nf-κb signaling reveal an almost identical skin appendage phenotype to these mouse mutants and analogy to human HED (Schmidt-Ullrich et al. 2001). These data show that Eda/Edar signaling is required for proper skin appendage development.

1.2.4 Fgf

Fibroblast growth factors (Fgf) are involved in various developmental processes like cell migration, differentiation, and proliferation (Ornitz and Itoh, 2001). Fgfs favor directional and reciprocal signaling across epithelial-mesenchymal boundaries (Hogan, 1999) and they function early in embryonic development by regulating mesoderm patterning and

establishing dorso-ventral axis. Later, Fgfs regulate limb induction and morphogenesis, bone formation, and midbrain-hindbrain patterning. In adults, they function also in tissue repair, injury responsies, and tranducing neuronal signals in the central and peripheral nervous system. (Celli et al. 1998; Ornitz and Itoh, 2001; Thisse and Thisse, 2005).

In human and mouse**,** 22 highly conserved Fgf proteins are known and the genes are widely located throughout the genome. Fgfs are secreted molecules with few exceptions and they can be divided into several subfamilies according to sequence similarity, receptor binding properties, and overlapping expression patterns. Fgfs signal through one of the four known Fgf receptors (Fgfr), transmembrane RTKs, the activation which requires HSPGs (Ornitz, 2000). The extracellular part of Fgfr consists of three immunoglobulin domains (Ig I-III) and a heparin-binding sequence. Two different Ig domain III forms, IIIb and IIIc, can be produced by tissue-specific alternative splicing of the receptor genes resulting in seven possible splice forms of receptors 1-3 and affecting the ligand-receptor binding specificity (Ornitz and Itoh, 2001; Thisse and Thisse, 2005).

The Fgfrs signal through tyrosine phosphorylation using different pathways of which the main is the Ras/Mitogen-activated protein (Map) kinase pathway depicted below. Fgfr is homodimerized upon binding of the Fgf-HSPG ligand complex which also induces the autophosphorylation of tyrosine residues in the intracellular part of the receptor. A membrane-anchored docking protein, Fgfr substrate 2α, interacts with the juxtamembrane domain of Fgfr and thus, is activated by phosphorylation, as well. This allows the signal to be mediated to GTP-binding Ras through growth factor receptor bound protein 2 and guanine nucleotide exchange factor, Sos. The signal is further transduced to serine/threonine-selective protein kinases Raf, Mek, and finally to Map kinases, which enter the nucleus to phosphorylate specific transcription factors of the Ets family, which activate the Fgf target genes. Fgf signaling is tightly regulated by a number of proteins that are coexpressed with Fgfs. Fgf signaling controls the expression of these regulators which include Sprouty and Sef. They antagonize Fgf pathway activity at the receptor level or downstream of it by forming negative feedback loops (Thisse and Thisse, 2005).

1.2.4.1 Fgf20

Fgf20 was identified over a decade ago and it forms a subgroup together with Fgf9 and Fgf16. They all have uncleaved bipartite secreted signal sequences required for secretion and when released, they function in paracrine manner. (Ohmachi et al. 2000; Ornitz and Itoh, 2001). Studies have implied that Fgf20 may signal through different Fgf receptors, like Fgfr2IIIc and FgfrIIIc (Hayashi et al. 2008; Ohmachi et al. 2003; Porntaveetus et al. 2011; Zhang et al. 2006).

The genomic location of Fgf20 gene has been detected to be in the Parkinson's disease risk locus (Scott et al. 2001; International Parkinson's Disease Genomics Consortium and Wellcome Trust Case Control Consortium 2, 2011). Studies have further revealed that it is expressed in the substantia nigra and appears to have neurotrophic and prosurvival actions on dopaminergic neurons in adult brain (Murase and McKay 2006; Ohmachi et al. 2000). Moreover, Fgf20 appears to regulate the proliferation and differentiation of myocardial cells, and has been implicated to be a transcriptional target of β-catenin, and has shown to be overexpressed in cancer cell lines (Chamarro et al. 2005; Lavine et al. 2005). Further, Fgf20

has been implicated in the regulation of the specification of inner ear sensory epithelium (Hayashi et al. 2008). Recently, analysis of *Fgf20*-null mice revealed deafness due to defects in the differentiation of the lateral compartment of the organ of Corti (Huh et al. 2012). Fin regeneration in zebrafish appears to require Fgf20 to guide the migration of the mesenchymal cells (Whitehead et al. 2005). Its role in skin appendage development is still not well known, although it was recently shown to be expressed in the primary and secondary signaling centers, the enamel knots, of the murine tooth (Porntaveetus et al. 2011, Häärä et al. 2012). Further, microarray results from our laboratory have suggested that *Fgf20* is an is a downstream target gene of Eda (Lefevbre et al. 2012) and *in vivo* it was shown that Fgf20 regulates tooth morphology by mediating Eda pathway signals (Häärä et al. 2012).

1.2.5 Tgf-β

The transforming growth factor β superfamily consists of 33 members in humans including glia-derived neurotrophic factor, nodal, the families of Bmps, growth and differentiation factors (Gdfs), Tgf-βs, and activins. The members of the superfamily appear to have similar structures and partly overlapping functions. The carboxy-terminal peptide region of Tgf-β superfamily proteins is processed to form the functional part involved in homodimer or heterodimer formation. The dimers are secreted and ligands recognize membrane-bound serine/threonine kinase receptors type I and II which further activate members of the Smad family of transcription factors. In addition to Smad, Tgf-β family members may activate other pathways, as well. (Heldin et al. 2009).

1.2.5.1 Bmp

Bmps form a large subgroup of 20 members (including Gdfs) within the Tgf-β superfamily. They are secreted proteins with seven conserved cysteines, acting as morphogens (Heldin et al. 2009). Diffusion distances are regulated by proteoglycans that recognize specific amino acids in the N-terminal part of Bmps. Bmps regulate cell migration, differentiation, apoptosis, and division (Hogan, 1996). As the name implies, Bmps are able to induce bone formation. In addition, analysis of transgenic mice with disrupted Bmp pathway genes has suggested that Bmp signaling is required early in embryonic patterning, regulating gastrulation, differentiation of lateral and heart mesoderm, and establishing left-right asymmetry (Kishigami and Mishina, 2005; Winnier et al. 1995). Deficiency of *Bmp2, Bmp4, Bmpr1a*, or *Smads 1, 4*, or *5* leads to early lethality (Botchkarev, 2002a; Hogan, 1996). Dysfuntion of pathway activity in adults, may cause defects in kidney, lungs, or bone, or may lead to cancer (Yanagita, 2005). Bmp signaling regulates organ development mainly through the canonical Smad pathway (see figure 2) but the signals may be transduced by Map kinases (Schmierer and Hill, 2007). In addition, Bmp pathway components have been shown to interact with other signaling pathways like Ca^{2+}/Calmodulin, Erk/Map kinase, and Jak-Stat (von Bubnoff and Cho, 2001).

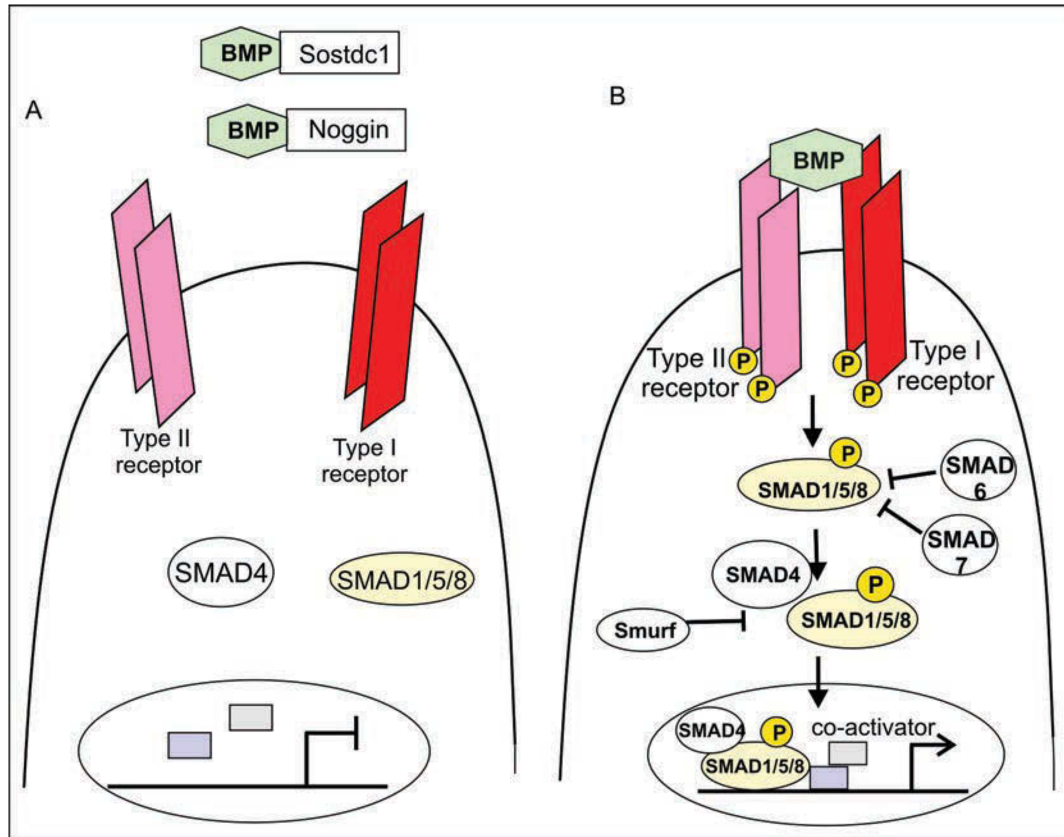

Figure 2. Bmp signaling through Smad-dependent pathway

(A) In the inactive state, Bmp ligands may be bound to antagonists, like Noggin and Sostdc1, and thus, be prevented from binding to their cognate receptors.
(B) Free Bmp ligand activates type I and type II receptors by binding to them and inducing type II receptor phosphorylation which further phosphorylates type I receptor. These events lead to phosphorylation of receptor regulated Smads (R-Smads), here Smad1, 5, and 8, which bind to co-Smad, Smad4, and the complex travels to the nucleus to regulate gene expression with other transcription factors, lsuch as Runx2 and Msx1. Bmp2 is able to activate Smad1, 5, and 8 whereas Bmp6 and Bmp7 induce only 1 and 5. The limited pools of Smad4 is also shared by Tgf-β and activin signaling. Intracellular inhibitors of the Bmp pathway are I-Smads, Smad6 and 7, which prevent R-smads from binding to co-smads. Their expression is induced by Bmp signaling. Smurf negatively regulates the intracellular R-smad pool and is involved in the degradation of type I receptors together with I-Smads. (Miyazono et al. 2005).

Bmps bind to three alternative subtypes of type I receptors which include the Bmp receptors (Bmpr) Ia , Ib, and the activin receptor (ActR) IA. For type II receptors there are three alternatives, as well: BmprII, ActRII, and ActRIIB. ActRs also function as signaling receptors for activins. Upon binding the ligand, dimeric type II receptor transphosphorylates the dimeric type I receptor which induces phosphorylation of its cytoplasmic substrates Smad 1, 5, and 8 which are different receptor-regulated Smads (R-Smads) from the ones (Smad 2/3) induced by activins, Tgf-βs, and Nodal. Activated Smad 1, 5, and 8 are released and bind to

Smad4 (common-partner Smad, co-Smad), which is also used by other Tgf-β signals. The established Smad complex (one co-Smad and two R-smads) translocates to the nucleus to interact with other transcription factors to induce target gene expression. (Schmierer and Hill, 2007). Among the most important target genes are *inhibitor of DNA binding* (*Id*) 1-4. They encode proteins with a helix-loop-helix dimerization domain in various cell types to promote cell proliferation and inhibit differentiation (Ogata et al. 1993; Yokota and Mori, 2002).

The Bmp pathway activity is tightly regulated at different levels by a number of antagonists through negative feedback loops (Miyazono, 2005). Extracellular antagonists that disturb the ligand-receptor interaction, include Noggin, Chordin, Cerberus, Gremlin, Dan, Cerberus-like protein 2, Twisted gastrulation, protein related to Dan and Cerberus, Sost, and Sostdc1 forming the subfamily of the cystine-knot superfamily (Yanagita, 2005; Zimmerman et al.1996) and Follistatin, a known activin inhibitor (Fainsod et al. 1997; Patel, 1998). These cystine-knot antagonists prevent a ligand from binding to its receptor by directly binding to Bmps (Yanagita, 2005). Other antagonists acting downstream include transmembrane inhibitor Bmp and activin membrane bound inhibitor, intracellular Smad6, Smad7, Smad1 antagonistic effector, and Smad ubiquitination regulatory factor-1 (Smurf1), which target R-Smads, and nuclear trancriptional repressors such as Tob, c-Ski and SnoN (Miyazono, 2005).

1.2.6 Sostdc1, the Bmp and Wnt pathway modulator

The vertebrate specific gene, *Sostdc1*, was discovered three times independently and named uterine sensitization-associated gene-1 (Usag-1), Wise, and Ectodin representing the *Rattus norvegicus, Xenopus,* and *Mus Musculus* orthologs, respectively (Itasaki et al. 2003; Laurikkala et al. 2003; Yanagita et al. 2004). According to its protein structure, with an N-terminal signal peptide required for its secretion and the conserved cysteine-knot motif, it was classified to belong to the Dan/cerberus family of Bmp antagonists, which is part of the cysteine-knot superfamily (Yanagita et al. 2004). Further, homology searches discovered Sost to be the closest homolog to Sostdc1 sharing 38% amino acid identity. *In vitro* studies have shown that Sost is able to inhibit both Bmp and Wnt pathway activities and defective *Sost* can cause sclerosteosis, a human syndrome of sclerosing skeletal dysplasia (Brunkow et al. 2001; Kusu et al. 2003; Li et al. 2005). Sostdc1 was observed to function as a Bmp antagonist and context-dependent Wnt modulator (Itasaki et al. 2003; Laurikkala et al. 2003; Yanagita et al. 2004) but despite the significant homology between Sost and Sostdc1 they do not appear to function identically in Bmp inhibition as they prefer different targets (Ellies et al. 2006).

The biochemical analysis of Sostdc1 has suggested that it may be glycosylated and that the secreted protein interacts with HSPGs but the significance of these characteristics remains still unsolved. Furthermore, there are controversial results whether Sostdc1 functions as a monomer or forms dimeric structure more typical to other Bmp antagonists. (Yanagita et al. 2004; Lintern et al. 2009). Studies have shown that the loop structures (or fingers; see figure 3) forming from the cysteine-knot domain serve to interact with Sostdc1's target molecules but different loop motifs are apparently used when Sostdc1 binds to Bmp4 or Wnt co-receptor Lrp5/6 (Lintern et al. 2009; see figures 1 and 2). Bmps have been shown to induce expression of *Sostdc1,* and Sostdc1 physically associates with Bmp2, 4, 6, and 7 and inhibits

their function, thus, forming a negative feedback loop. It has been reported, that Sostdc1 has lower affinity to Bmp ligands than Noggin (Laurikkala et al. 2003; Lintern et al. 2009; Mou et al. 2006; Yanagita et al. 2004). Sostdc1 binds to epidermal growth factor (Egf) domains 1 and 2 of co-receptors Lrp5/6 which are different than the ones Dkk1 recognizes and according to *in vitro* studies Bmp-4 is not able to interfere the Sostdc1- Lrp5/6 interaction. (Lintern et al. 2009). Sostdc1 has been shown to compete with Wnt proteins 1, 3, 8, and 10b for binding to the co-receptor (Beaudoin III et al. 2005; Blish et al. 2008; Itasaki et al. 2003). Moreover, Lrp4, apparently functioning as a negative regulator of Wnt activity, has been shown to interact with Sostdc1, as well (Ohazama et al. 2008;). Another study has proposed that Sostdc1 may inhibit Wnt signaling by sequestering Lrp6 from the cell surface when the antagonist is present in the endoplasmic reticulum instead of being secreted (Guidato and Itasaki, 2007).

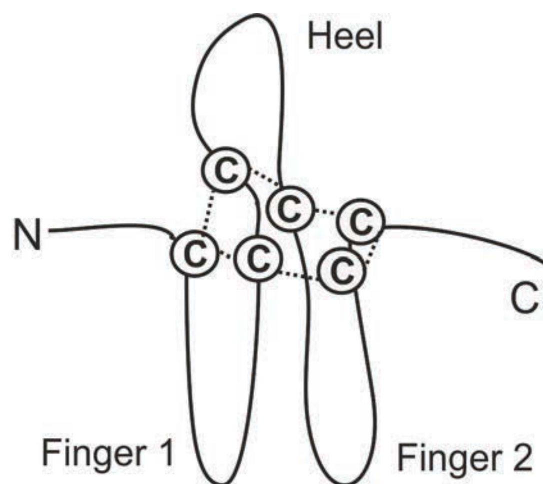

Figure 3. Schematic model of Sostdc1 protein.

Sostdc1 protein reveals three loop structures: heel, finger 1, and finger 2. Six cysteines (C) form a knot by covalent bonds in the center of the protein thus, connecting the looped structures. N=N-terminus; C=C-terminus.

Gene or protein expression analyses of Sostdc1 have revealed its localization to mouse head and trunk skin ectoderm prior to hair placode formation, interdigital tissues, in embryonic and postnatal hair follicles, vibrissae, teeth, kidney, and in developing tongue papillae and testis (Ahn et al. 2010; Laurikkala et al. 2003; Munne et al. 2009; O'Shaughnessy et al. 2004; Yanagita et al. 2004). Furthermore, *Sostdc1* has a unique expression pattern in hair placodes and developing teeth. It is epithelially localized to the immediate surroundings of hair primordia being absent from the developing organ itself (Laurikkala et al. 2003). In developing teeth, it is absent from the signalling centers of molars and incisors, the enamel knots, showing mainly mesenchymal localization but it is also detected in the tooth epithelium (Laurikkala et al. 2003; Munne et al. 2009). Sostdc1 has also been implicated in

cancer, showing reduced expression in Wilms tumors and in renal and breast cancer cells (Blish et al. 2008; Ohshima et al. 2009; Clausen et al. 2010).

The *in vivo* effects of *Sostdc1* have been characterized in kidney, especially in renal injuries where it functions as a Bmp-7 antagonist (Yanagita et al. 2006; Tanaka et al. 2008) and to rather large extent in mouse tooth formation as an inhibitor of Bmp and Wnt signaling (Kassai et al. 2005; Murashima-Suginami et al. 2007; Murashima-Suginami et al. 2008; Munne et al. 2009; Ohazama et al. 2008; Ahn et al. 2010; Cho et al. 2011). The tooth phenotype of *Sostdc1*-null mice was characterized by Kassai et al. (2005) and the mice were shown to have extra teeth, changed cusp patterns, and fused molars which showed sensitivity to excess Bmp4 when grown in vitro. Dkk1 and Noggin were subsequently shown to prevent the formation of extra *Sostdc1*-null incisors in tooth cultures (Munne et al. 2009). Another study revealed that Sostdc1 defiency resulted in increased nuclear β-catenin levels together with enhanced Bmp activity, implying that antagonism of both Bmp and Wnt signaling is required for regulating the tooth number (Murashima-Suginami et al. 2008). Interestingly, *Lrp4*-null mice were shown to have similar tooth phenotype to that of *Sostdc1* mutants and it was speculated that in wild-type conditions, Sostdc1 binds Bmp and that they form a tertiary complex with Lrp4 that negatively regulates Wnt signaling (Ohazama et al. 2008). In that study, Sostdc1-mediated Bmp inhibition in the absence of Lrp4 was not detected. Recently, *in vivo* evidence was shown for Wnt signaling to function in Sostdc1-regulated tooth development, as mice deficient with *Lrp5 and Lrp6* rescued the *Sostdc1*-null tooth phenotype (Ahn et al. 2010). Further, it was suggested that the regulatory mechanism may involve Shh as a negative feedback regulator for Wnt perhaps through indirect induction of *Dkk1* (Ahn et al. 2010; Cho et al. 2011).

1.3 Hair function and development

Hair development begins during embryogenesis and continues postnatally. A strict genetic program and the signaling molecules encoded by this program underlie and guide hair development. Systemic endocrine factors, such as estrogens and androgens, have been elucidated to govern hair growth, as well (Kaufman, 2002; Plikus and Chuong, 2008). Hairs are composed of filamentous biomaterial, keratins, and they grow from follicles located in the dermis. Found in mammals, hairs play a role in sensation, heat loss, and provide protection, but to humans, they also have a cosmetic relevance. In humans, there are known inherited diseases in which there are major hair abnormalities characterized by defective hair, hair follicle structure, hair pigmentation, or hair loss (alopecia) (Goldsmith, 1994). To understand hair development, the pathophysiology, and molecular basis of hair-related diseases and to test novel therapies for those diseases, animal models, like dogs, rats, and mice, have been used. These models show either spontaneous mutations (dogs, rats, and mice) or are produced by transgenic or knockout technology (mice). (Sundberg, 1994; Drögemüller et al. 2008; Moura and Cirio, 2004). In what follows, I will focus on mouse hair types and structure, hair placode patterning and molecular regulation, embryonic hair morphogenesis, and postnatal hair cycling.

1.3.1 Mouse hair types and structure

The overall morphology of hair shafts is similar but variability is seen in different sizes and shapes. The molecular basis for these variations is not well known, though. Mouse hairs differ in size and anatomical location and are classified to eight types, of which many are rarely examined like cilia (eyelashes) in different mouse transgenics or spontaneously mutated strains (Sundberg and Hogan, 1994). The four pelage (or trunk) hair types (see figure 4), comprising straight guards and awls and bended auchenes and zigzags (Dry, 1926), have been the most intensively studied. The development of these different trunk hair follicle types is induced in three separate waves: Guard hair follicles appear in primary wave at E14.0 and the second (E15.5-E16.5) and third (~E18) hair waves give rise to the awl/auchene and zigzag placodes, respectively, in the dorsal skin (Mann, 1962; Schmidt-Ullrich and Paus, 2005). The longest hair filaments (> 9 mm), guards, comprise usually 2-4 % of the fur hair number. The fur undercoat is formed by short awls (25-30 %), auchenes (5-10 %) with one bend, and the most general hair fibers (60-70 %), zigzags, with three to four bends. (Schlake, 2007). The molecular basis for the production of the four different pelage hair shapes is still largely unknown but studies suggest that there are variations in genes required for specific hair types for example, the Eda pathway regulating the formation of bended zigzags (Duverger and Morasso, 2009; Schlake, 2007).

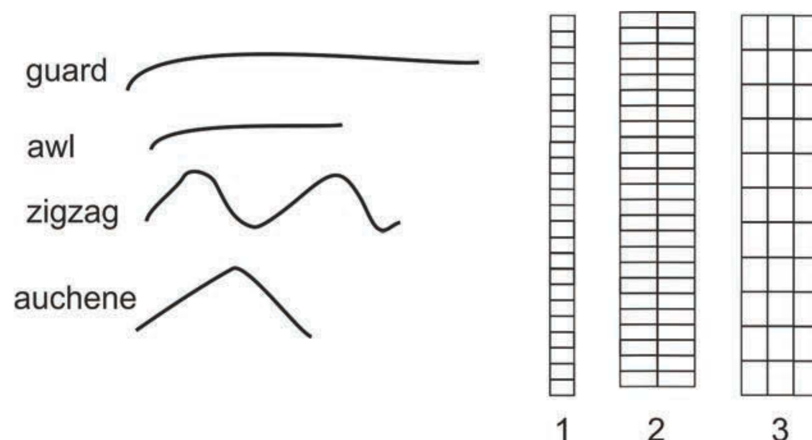

Figure 4. Pelage hair types and their inner structure

Schematic drawings of the four pelage hair types on the left: straight guard and awls differ by length and bended zigzags and auchnenes can be distinguished by the number of bends in hair filament.Hair types can also be classified according to the number of rows formed by medullary cells: zigzags contain one (1), guard hairs two (2) and awls may consist of two or three (3) rows.

Vibrissae (also known as tactile or sensory hairs) are specialized somatosensory organs located in blood-filled sinuses with neuronal innervations. Three types of vibrissae are known: primary, secondary, and supernumerary vibrissae (Duverger and Morasso, 2009). Mystacial or primary vibrissae (whiskers) which are organized precisely by number and location are found in the muzzle. Interesingly, it has been discovered that the peripheral patterning of mystacial vibrissae is also responsible for establishing the pattern of barrels, the segregated columns in the somatosensory cortex (Van der Loos et al. 1984; Ohsaki and Nakamura, 2006). Secondary vibrissae with specific number consist of postoral, suborbital, and supraorbital vibrissae in the snout region, inter-ramal sensory hairs located in the mandibular skin, and ulnar-carpal vibrissae in limbal skin (Van der Loos et al. 1984). There are mouse strain-specific organizational patterns of both primary and secondary vibrissae caused by genetic differences which also cause the appearance of supernumerary mystacial vibrissae within the vibrissa pad region (Van der Loos et al. 1986). Other hair types include the short hair fibers protruding from the tail and ear skin. There are also anatomical regions devoid of hairs (glabrous regions), such as the ventral palms and nipple. (Sundberg and Hogan, 1994). The molecular mechanisms behind the regional specificity are still incompletely understood. It has been speculated that the distinct inductive potential of the mesenchyme along the body axes could determine these differences perhaps through a specific HOX code (Chang et al. 2002; Duverger and Morasso, 2009).

Three distinct cell lineages, cuticle, cortex, and medulla contribute to hair filament's structure and shape. The cuticle forms the hair surface and the cortex produces keratins required for hair rigidity. The regulation of keratin expression is not fully understood but it is believed to involve the canonical Wnt signaling, Wnt3, and the Bmp pathway, the latter further regulating *Foxn1* and *Hoxc13* expression (Kulessa et al. 2000; Millar et al. 1999; Schlake, 2007; Zhou et al. 1995). The medulla structure consists of columns formed by shrunken medullary cells separated by air spaces, a feature that is not observed in human hairs (Sundberg and Hogan 1994; Schlake, 2007). The number of columns varies among the different hair types (see figure 4) and are easily detectable under microscope, which is used for classifying the hair filaments e.g. guards, awls, zigzags have two, two to three, and one rows of medullary cells, respectively (Dry, 1926).

1.3.2 Hair placode formation

Hair placodes appear in wave-like fashion rather than all of them arising simultaneously. Before clear morphological signs of local groups of elongated epithelial cells, skin shows molecular patterning in the E13.5 lateral ectoderm at sites of pre-placodes (Bazzi et al. 2007; Fliniaux et al. 2008; Schmidt-Ullrich and Paus, 2005). By E14.0 both the molecular (see figure 5) and morphological patterning of the primary hair placodes is visible largely in the whole dorsal skin. Hair placodes of ventral skin, dorsal midline, and paw skin appear later. The "vibrissa wave" begins before pelage hair development is initiated. First, the mystacial vibrissal placode is visible around E12.0 and by E14.5 the full pattern has formed with five horizontal rows (rostrocaudal) and one vertical (dorsoventral) row caudally with four vibrissal follicles (van Exan and Hardy, 1980). The vibrissa wave proceeds from caudal to rostral borders and in a ventral to dorsal direction within the defined vibrissa pad region (van Exan and Hardy, 1980). Placode formation and the following morphogenesis of different pelage hair types

and vibrissal follicles is thought to be largely regulated by similar molecular mechanisms, but they appear to have a distinct genetic basis (table 1; Duverger and Morasso, 2009).

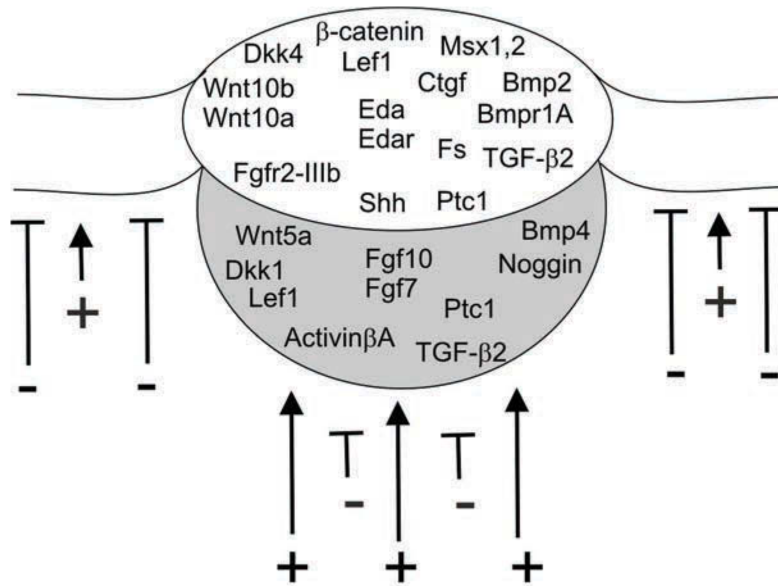

Figure 5. Hair placode associated gene expression.

Schematic view of E14.0-E14.5 hair placode with marker genes expressed in the placode epithelium or dermal condensate. Members from Tgfß, Wnt, and Fgf families are expressed in both compartments but the Eda pathway ligand and receptor show epithelial localization. Hair placodes contain both placode promoters (e.g. Wnt10b and Eda/Edar) and inhibitors (e.g. Bmp2,4 and Dkk1,4). At the placode site, positive growth promoting signals (+) overcome the negative (-) effects from placode inhibitors, which promote interfollicular epithelium formation.

Based on tissue recombination experiments, it is believed that the first signal leading to hair placode or feather bud formation arises from mesenchyme (Hardy, 1992; Dhouailly 1973). For these studies, the enzymatically separated mouse and chick skin dermis and epidermis from various body regions were recombined either in a heterospecific (mouse vs. chick) or a homospecific (mouse vs. mouse) manner. In the latter case, homologous or heterologous (explants from different body regions e.g snout, dorsum, or non-haired skin area recombined in several ways) combinations were examined. The studies showed that patterned (contains dermal condensates) dermis is able to induce hairs or feathers in non-patterned mouse or chick epidermis, respectively, dissected from normally haired or feathered skin region. Furthermore, patterned (containing placodes or buds) epidermis derived from chick or mouse was also able to initiate skin appendage development when combined to non-patterned dermis from hair or feather forming area. These results highlight

the inductive nature of dermis, but do not demonstrate where the first inductive signal arises. Interestingly, when dermis was dissected from a normally non-haired or non-feathered skin region (glabrous area), it was not able to induce epidermis from a normally haired/feathered region to initiate skin appendage development. Reciprocal combination of tissues of the latter experiment, however, resulted in cutaneous appendage formation in glabrous epidermis responding to signals from patterned dermis. (Dhouailly, 1973; Kollar, 1966; Kollar 1970). Based on these two latter experiments, it is thought that epidermis does not have intrinsic potential to initiate hair of feather development but the first signal(s) rather derive from dermis. Still, the first dermal message lacks identity. Also the behaviour of the first signal whether it is uniformly expressed or provided as patterned signal has been under study but no answer to this question has been found (Jiang et al. 1999; Millar, 2002).

The generation of dermis appears to require canonical Wnt signals both in mouse and chick (Atit et al. 2006; Noramly et al. 1999), but Wnt activity is also present prior to and during initiation of hair follicle development (Fliniaux et al. 2008; Zhang et al. 2009). Wnt reporter mice have shown that Wnt signaling activity is present in the upper dermis of E12.5-E13.5 skin (Zhang et al. 2009). Upon hair follicle initiation, Wnt activity it is localized to E14.5 placodes and the underlying dermal condensates (DasGupta and Fuchs, 1999; Zhang et al. 2009). Furthermore, gene expression analysis of Wnt pathway components have revealed that E14.5 skin epithelium shows the expression of several Wnt ligand genes namely *Wnts 3, 4, 7a, 7b, 10a, 10b, and 16* (Reddy et al. 2001). The most convincing *in vivo* data for supporting the idea that Wnt signaling lies upstream of most other pathways around initiation of hair development has been shown by analysis of knockout and transgenic mice with silenced Wnt signaling. Absence of epithelial *β-catenin* and overexpression of *Dkk1* leads to absence of trunk and vibrissa placodes. (Huelsken et al. 2001; Andl et al. 2002; Zhang et al. 2009). Other Dkk family members like Dkk2 and 4 have also been shown to be able to inhibit placode formation (Cui et al. 2010; Bazzi et al. 2007; Krupnik et al. 1999; Sick et al. 2006). *Lef1* deficiency causes loss of vibrissa placodes, as well, but few pelage hair placodes are observed (van Genderen et al. 1994). Further, the skin-specific knockout of *Lgr4*, a gene involved in the positive regulation of Wnt signaling, leads to the absence of primary hair development (Mohri et al. 2008). Moreover, stabilized β-catenin in skin epithelium is able to induce postnatal *de novo* hair formation from interfollicular dermis or pre-existing follicles (Gat et al. 1998; LoCelso et al. 2003; Silva-Vargas et al. 2005; Zhou et al. 1995). However, β-*catenin* gain-of-function mice did not reveal embryonic hair phenotype (Gat et al. 1998). For comparison, in chick feathers, canonical Wnt pathway appears to affect early development, as stabilized β-catenin causes ectopic feather bud formation (Noramly et al. 1999).

In addition to canonical Wnt signaling, other genes and pathways have been shown to affect hair placode formation, as well, but the hierarchy of these signals is still unclear. During placode formation *Eda* is expressed in the ectoderm between placodes and whereas its receptor *Edar* is present in the placodes. Inactivation of the Eda pathway activity in *tabby* (*Eda-/-*), *downless* (*Edar-/-*), or *crinkled* (*Edaradd-/-*) mice results in the absence of primary hair placodes (and loss of guard hairs) but the subsequent hair waves are spared (Headon and Overbeek, 1999). A similar hair phenotype is observed in mice with constitutive inhibition of Nf-κb signalling (Schmidt-Ullrich et al. 2001). *In vivo* or *in vitro* overexpression of *Eda-A1* leads to the formation of enlarged hair placodes (Mustonen et al. 2004). Lack of Troy, a homologous receptor to Edar, does not affect hair formation or development but the

simultaneous absence of both receptors results in a loss of both primary and secondary hair follicles (Pispa et al. 2008).

Earlier studies have shown that Fgf signaling through Fgfr2IIIb promotes hair induction *in vivo* (Petiot et al. 2003). However, recent *in vitro* tissue cultures showed Fgf7 (also known as keratinocyte growth factor) and Egf to promote interfollicular epidermis formation at the expense of hair follicles, presumably signaling through Fgfr2IIIb and Egf receptor, respectively, which were detected to be downregulated upon placode formation, (Richardson et al. 2009a). In addition, a recent conditional combined knockout of *Fgfr1* and *Fgfr2* resulted in the formation of hair follicles with normal density but during postnatal hair cycling the hair filaments were progressively lost (Yang et al. 2010). The function of mesenchymal Fgfs in hair development is not known.

As there appear to be several placode promoters, there is also requirement for repressors of hair induction or promoters of interfollicular cell fate in order to achieve a regular spacing of hair placodes as hexagonal arrays. In chicks and mice, Bmp2 and Bmp4 have been suggested to repress placodal fate (Blessing et al. 1993; Botchkarev et al. 1999; Jung et al. 1998; Noramly and Morgan, 1998), and they have been shown to act downstream of β-catenin signaling (Huelsken et al. 2001). Further, knockout of the Bmp inhibitor *Noggin* in mice, leads to the appearance of primary hair placodes which are later developmentally arrested, and to the absence of secondary hair placodes with upregulation of *Bmp2* and *Bmp4* (Botchkarev et al. 2002b). In addition, in mice and chicks overexpression of *Noggin* also causes ectopic hair follicles or feather buds (Noramly and Morgan, 1998; Botchkarev et al. 1999; Jiang et al. 1999). Tgf-β1 has been shown to negatively regulate hair follicle development *in vitro,* and mice with *Tgf-β1* deficiency show slightly advanced hair follicle formation (Foitzik et al. 1999). On the other hand, in mice, simultaneous inactivation of the placodal homeobox-containing genes *Msx1* and *Msx2* whose expression is thought to be regulated by the Bmp pathway or loss of *Tgf-β2* lead to reduced number of hair follicles (Foitzik et al. 1999; Satokata et al. 2000). Thus, Tgf-β family members may mediate both positive and negative signals during hair placode formation.

1.3.3 Molecular control of placode patterning

The appearance of regular arrays of placodes has been tentatively explained by mathematical modeling. The reaction-diffusion model, which shows how patterns arise autonomously without prepattern, was developed 60 years ago by Turing (1952) to explain for example the morphogenesis of Hydra but it has aslo been used as a basis to model the skin appendage patterning (Kondo and Miura, 2010; Salazar-Ciudad and Jernvall, 2002; Sick et al. 2006; Wang et al. 2006; Michon et al. 2008). The reaction diffusion model is based on differencies in the diffusion properties of an activator and an inhibitor and their reciprocal signaling loops. The reaction-diffusion model assumes that the activator induces its own expression as well as its inhibitor. The inhibitor diffuses faster and more widely than the activator and thus, the target cells behave according to the signals they get. In the case of skin appendages cells become placodal cells at sites of high activator levels or obtain interplacodal fate when activator/inhibitor relation is low. In general, several signals are first expressed at low level throughout the ectoderm but upon placode induction get localized. Epithelial/mesenchymal *Lef1* and epithelial *Wnt10b* have been shown to have evenly spread expression pattern but

then become subsequently upregulated at the sites of placode formation (Reddy et al. 2001; Zhou et al. 1995). In hair placode formation, the assumed essential roles played by Wnt signaling and its inhibitors Dkks has raised the suggestion that they form a suitable reaction-diffusion pair (Andl et al. 2002; Sick et al. 2006).

Computer modeling used for predicting how placode patterning behaves during primary hair follicle formation has shown that elevated Dkk levels increase the interfollicular spacing between hair placodes and opposite effect is observed when the Wnt activity is increased as the placode size itself increases. Further, later hair waves were predicted to behave by forming hair follicle clusters when Dkk levels were increased. These computer-based models were tested *in vivo*, as well, using transgenic *Dkk1*, *Dkk2*, and *Dkk4* overexpressing mice whose placode patterning showed similar behaviour as the predictions (Sick et al. 2006).

In addition to Wnt-Dkk interplay, other activator-inhibitor pairs forming reciprocal signaling loops have been implied to guide the hair follicle patterning, as well. Edar is expressed ubiquitiously prior to hair follicle development but is upregulated at sites of hair placodes upon formation (Headon and Overbeek, 1999). Primary hair follicles have been shown to depend on Eda/Edar activity as well as on Wnt signals (Headon and Overbeek, 1999; Huelsken et al. 2001). *In vitro* tissue culture studies have shown an interplay between Edar and Bmp signaling that was suggested to be responsible for primary hair placode patterning involving restriction of responsiveness of the target cell to a widely available signal as a key mechanism in this model (Mou et al. 2006). Eda signaling was shown to indirectly induce gene expression of Bmp ligands 4 and 7, and Bmp signaling was revealed in turn to inhibit *Edar* expression at a distance from the forming follicle by inhibiting responding cells from attaining a placodal fate. Further, Eda pathway was shown to activate *Edar* and *Ctgf*, the latter encoding a protein with Bmp inhibitor activity within the placode, thus suggesting a more complex regulatory network than the one suggested for Wnt and Dkk. (Mou et al. 2006).

1.3.4 From placode to mature follicle

The schematic view of embryonic hair follicle morphogenesis is depicted in figure 6. Once the placode is formed it is believed to tranduce the first epithelial signal to underlying mesenchymal fibroblasts causing the formation of the dermal condensate. This becomes histologically evident at E14.0 (DasGupta and Fuchs, 1999; Hardy, 1992; Huelsken et al. 2001). Interestingly, histological analysis of vibrissal development has shown a morphological appearance of dermal condensates prior to epithelial thickenings (Wessells and Roassner, 1965) but it has not been confirmed molecularly that the mesenchyme is patterned first. In pelage hairs, it is thought that the morphological signs of placode and dermal condensate formation appear to arise simultaneously (Sengel, 1976). Mice lacking Eda/Edar/Nf-κb signaling show pre-placodes, which do not develop further, but no signs of dermal condensate formation is detected at this point (Schmidt-Ullrich et al. 2006) suggesting that the epidermal compartment of the placode may arise first. However, currently there are no other known mouse models showing the presence of one compartment in the absence of the other. The dermal condensation can be distinguished also by the expression of various markers like Sox2 (SRY-box containing gene 2), Syndecan1, Cd44 antigen, *Dkk1*, *activinβA*, *Bmp4*, Bmp

transcriptional target gene *cyclin-dependent kinase inhibitor* (*p21*), and Noggin, which are absent from the placode epithelium (Millar et al. 2002; Driskell et al. 2008; Richardson et al. 2009a; Richardson et al. 2009b; Underhill, 1993).

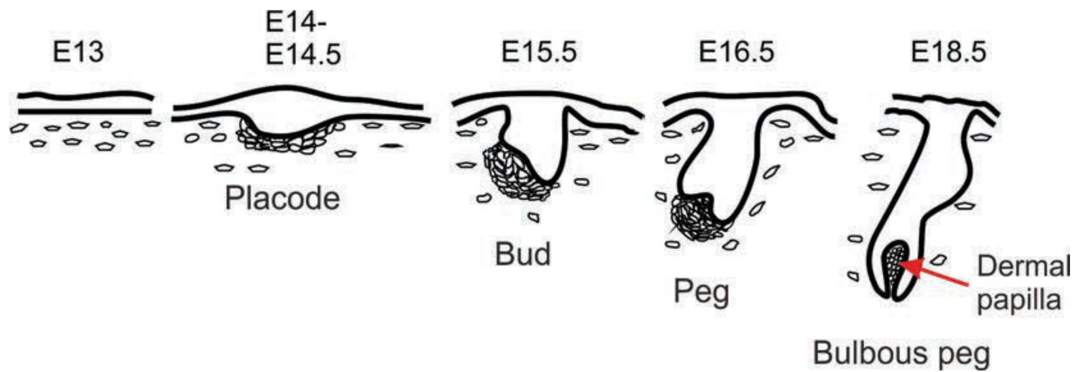

Figure 6. Embryonic pelage hair follicle morphogenesis

The hair follicle morphogenesis depicted above is morphologically similar to mystacial vibirissae and pelage hair follicles but temporal development is different. At E13.0, dorsal skin epithelium is flat overlying evenly distributed mesenchymal fibroblasts. Pelage hair pre-placodes appear on ectodermal flanks around E13.5 but become morphologically more visible by E14.0. At this time the thickened and sligthly invaginated placode is associated with dermal thickening formed by condensed fibroblasts. The epithelial-mesenchymal reciprocal signaling guides the following morphogenetic steps leading to hair bud formation by E15.5 which further invaginates into the dermis and folds to obtain the follicle shape prior to birth. The dermal condensate is gradually captured by the proximal hair germ tip from E16 onwards to form the dermal papilla (arrow) by E18.5.

It is generally thought that the dermal condensate sends back the second mesenchymal signal inducing the placode which grows through proliferation and penetrates deeper into the dermis (Hardy, 1992). *In vitro* studies have suggested that downregulation of E-cadherin in adherens junctions appears at the site of hair bud formation is correlated with increased levels of nuclear β-catenin, Lef1, and *TOP-gal* Wnt reporter gene (Jamora et al. 2003) and that this negative regulation of E-cadherin is due to direct actions of β-catenin and Lef1 (Jamora et al. 2003). Hair bud formation also requires epithelial expression of *Shh* which appears to exert its function through Gli2, as disruption of either gene leads to similar developmental arrest at the early hair bud stage and only epithelial Gli2 activator can rescue *Shh*-null phenotype (Chiang et al. 1999; St.Jaques et al. 1998; Mill et al. 2003). Shh induces the accumulation of *Ptc1* and *Gli1* both in placode epithelium and mesenchyme thus enabling reciprocal crosstalk between the two compartments (Dahmane et al. 1997; Platt., 1997; Ghali et al. 1999). The Eda pathway has been shown to be upstream of *Shh* which induces expression of

cyclinD1, a well-characterized target of the canonical Wnt pathway, and cyclinD2, which is required for cell division (Fliniaux et al. 2008; Mill et al. 2003; Schmidt-Ullrich et al. 2006). Shh has also been shown to be responsible for *Wnt5a* expression in the dermal condensate (Reddy et al. 2001). Wnt signaling is likely also required for *Shh* expression (Huelsken et al. 2001; Silva-Vargas et al. 2005). Adhesion molecules, like α-catenin, and basement membrane components have been shown to play a role in the hair germ downgrowth (Vasioukhin et al. 2001). The primary laminin isoform of embryonic hair follicle is laminin 511, and its genetical ablation leads to developmental arrest around E16.5 at hair peg stage (DeRuen et al. 2010). Further, laminin 511's primary receptor in the epithelium, β1 integrin, is required for remodeling the basement membrane and hair germ invagination, as well. (Brakebusch et al. 2000; Raghavan et al. 2000).

The further invagination of the epithelial bud forms a hair peg which encases the condensed dermal cells thus leading to the formation of a dermal papilla. Other fibroblasts form a dermal sheath lining the invaginating follicles on both sides. Loss-of-function of platelet derived growth factor a (Pdgfa) causes dermal papillae with reduced size and defects in dermal sheats (Karlsson et al. 1999). The steps following bulbous peg stage around E18 involves the formation of the seven cell layers present in the mature hair follicle (Millar, 2002). Differentiation of the inner root sheath (IRS), which consists of three cell layers including Huxley, Henle, and cuticle, and hair shaft, is regulated at least by Notch signaling from the hair matrix cells, located at the base of hair bulb (Powell et al. 1998; Millar, 2002). The outer root sheath (ORS), the contiguous layer of the skin basal cells, envelopes the IRS and hair shaft. Its differentiation requires the transcription factor Sox9 (Vidal et al. 2005). Analyses of transgenic and knockout mice have suggested that activinβA signaling from the dermal condensate and its epithelially localized inhibitor Follistatin are required to modulate hair morphogenesis (McDowell et al. 2008; Nakamura et al. 2003). The hair follicle continues to mature still postnatally forming a complex but organized mini-organ by ~P6-8 after which the hair filaments exit the skin surface (see figure 7; Alonso and Fuchs, 2006). Wnt signaling has to be tightly regulated throughout the morphogenesis of hair follicle as recently it was also shown that loss of Apccd1, a membrane-tethered glycoprotein and a novel Wnt antagonist that interacts with Lrp5, leads to the formation of miniature hair follicles in humans, a condition known as hereditary hypotrichosis simplex (Shimomura et al. 2010).

The polarity of hair follicles, which is established during embryogenesis and results in hair filaments pointing posteriorly, may be regulated by Wnt signaling which activates Shh downstream, as stabilized canonical Wnt pathway results in misangled hair follicles and change of asymmetrical expression pattern of Shh to symmetric (Gat et al. 1998; Devenport and Fuchs, 2008; Kuraguchi et al. 2006; Millar et al. 1997; Zhou et al. 1995). PCP pathway signaling through Fz6 receptor may regulate the polarity of hair follicles, as well (Wang et al. 2006). *Fz6*-null mice reveal embryonic phenotype of hair follicle orientations that appear to be randomized; however during the first week of postnatal development the follicles reorient (Wang et al. 2006).

Further, during early embryonic hair development, two other core PCP proteins not involved in the canonical Wnt pathway, Vang like 2 and Celsr1 (cadherin, EGF LAG seven-pass G-type receptor 1), have been detected to be asymmetrically localized along the anterior-posterior axis of hair germs. In addition, mouse models with point mutations in Vang like 2 and Celsr1 genes formed mis-angled hair follicles due to lost anterior-posterior polarization. *In vitro* it was also shown that interaction of Vang like 2,

Celsr1, and Fz6 is required during the establishment of hair follicle polarity. (Devenport and Fuchs, 2009).

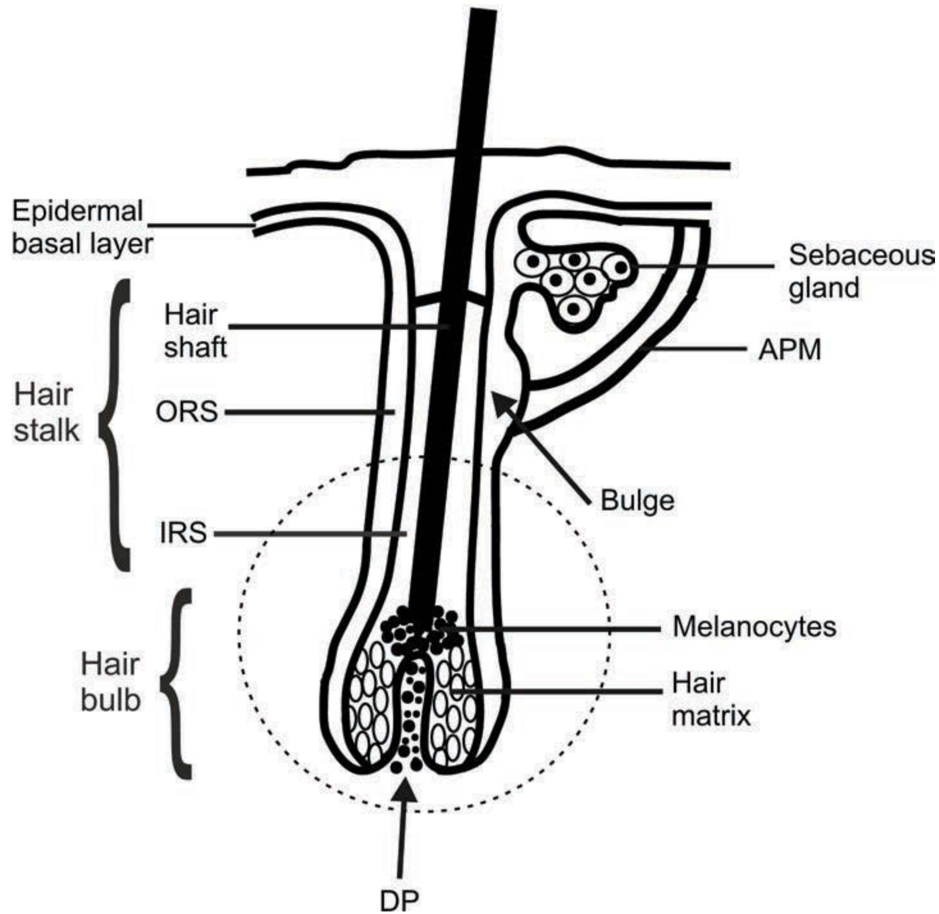

Figure 7. Mature hair follicle

The hair follicle matures early in postnatal life. The follicle epithelium with seven cell layers from outermost to innermost consists of ORS (covered by dermal sheath) which is contiguous with the epidermal basal layer, IRS (with Henley, Huxley, and cuticle layers), and hair shaft (with cuticle, cortex, and medulla layers). The hair bulb (marked by a bracket) is the proximal structural part consisting of the follicular matrix, which is responsible of forming the hair shaft layers, melanocytes, which provide hair pigments, and the mesenchymal dermal papilla. The dotted circle defines the follicle portion which is involved in the repeated regression and growth phases during postnatal hair cycling. Above this cycling portion, the hair stem cell niche (bulge) is attached to arrector pili muscle (APM), which is responsible for lifting/ lowering the hair filaments in response to temperature changes. The hair stalk lies above the bulb and overlaps both cycling and permanent portion of the follicle. Sebaceous gland is located most distally to the other compartments. Guard hairs have two of these glandular structures, whereas the other pelage hair types have only one.

1.3.5 Postnatal hair cycling

The hair coat is supplied by new hairs, thus, hair follicles constantly cycle between rest and growth, a process lasting through the animal's life-time (see figure 8). The cycling tends to follow precise time schedule but there are variations according to mouse strain (genetic background), age, sex, and environmental and nutritional factors. The three main murine cycle stages involve telogen (resting phase), anagen (growth phase), and catagen (regressive phase), of which the last two are further divided into eight stages (Dry, 1926; Müller-Rover et al. 2001). All the distinct stages can be classified by histological and histochemical/ immunohistochemical stainings. Gross classification using pigmented mice can be performed by analysing the pigmentation and thickness of the dorsal skin, as both increase during anagen progression and decrease by telogen. (Müller-Rover et al. 2001). The hair cycle of mystacial vibrissae differs from that of pelage hair follicles showing longer anagen and direct transit of whisker follicles from midcatagen to growth stage. Regulation of activation and stem cell movements are most probably shared features among vibrissal and pelage hair follicles. (Blanpain and Fuchs, 2006)

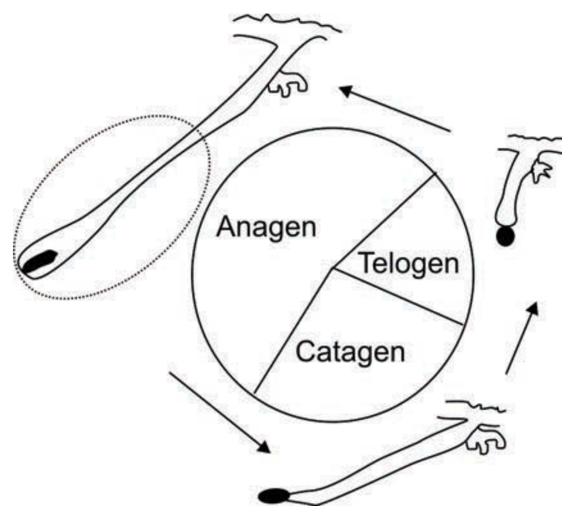

Figure 8. Postnatal hair cycling.

The hair cycles throughout animal's life showing three morphologically distinct stages. The catagen, involves apoptosis which degrades the cycling portion (defined by a dotted circle) of the mature anagen follicle, sparing the dermal papilla which is dragged upwards to be located in close proximity with the bulge (stem cell niche) at the resting stage, telogen. Telogen hair follicle is short and quiet. Competition between growth promoting and inhibiting signals regulate the maintenance of resting phase and the entry into next growth phase, anagen. Crosstalk between dermal papilla and bulge regulates the anagen initiation. The duration of anagen is longest and during this time the cycling portion is regenerated and new hair filament is produced.

Hair cycling occurs in synchronized wave pattern in anterior to posterior direction but as the mouse gets older, mosaic patterns showing asymmetry in the progess of hair cycling increases in neighbouring hair follicle areas (Plikus and Chuong, 2008; Plikus et al. 2008). Approximately two weeks after birth, mature hair follicles enter the first cycle through catagen which lasts only couple of days and is driven by apoptosis. The cycling portion covering two-thirds of the mature follicle is largely lost but dermal papilla is spared by the destructive actions and is draged up towards the permanent portion of the hair follicle by epithelial strand, which is eliminated by telogen. The telogen hair follicles with sebaceous gland is recognized by a ball-shaped dermal papilla located in juxtaposition to the bottom of the anchored club hair, called secondary hair germ. The first hair follicle dormancy lasts only one to two days but the following telogen is longer than two weeks. (Müller-Rover et al. 2001). The following anagen involves re-growth of lower part of the hair follicle, differentiation of IRS and generation of a new hair filament. The full-length anagen follicles are distinguished as straigth and long structures which invaginate deep into the subcutaneous layers of skin. The anagen length correlates to the hair length and it depends on the proliferation and differentiation capacity of the matrix cells. (Alonso and Fuchs, 2006). As the new hair shaft is formed during anagen, the old hair fiber known as club hair from the previous cycle remains attached to the permanent portion of the follicle but is later shed away in exogen phase. One follicle may have several club hairs attached to it (Alonso and Fuchs, 2006).

The transition from telogen to anagen involves activation of the hair follicle stem cell niche known as a bulge, which is located in the permanent portion below sebaceous gland, next to the bottom of club hair and above dermal papilla and secondary hair germ (Blanpain and Fuchs, 2006; Cotsarelis et al. 1990; Greco et al. 2009; Jaks et al. 2008; Nowak et al. 2009; Oshima et al. 2001; Taylor et al. 2000; Woo and Oro, 2011). The bulge becomes morphologically distinguishable when one or two bulge stem cells begin to proliferate presumably in response to dermal papilla signals to form the transit-amplifying cells and thus, an additional layer is included to this stem cell niche (Alonso and Fuchs, 2006; Sun et al. 1991). A secondary hair germ, lying between bulge and dermal papilla, responds to the signals from the dermal papilla upon which the bulge gets activated as well, leading to rapid proliferation and fueling the early steps of anagen (Greco et al. 2009). The exiting proliferating bulge stem cells migrate various distances along the ORS and are thought to have distinct fates when regenerating the lower cycling portion of hair follicle (Tumbar et al. 2004). Recent studies have enlightened the dynamics of the bulge stem cells during regeneration of hair by pulse-chase and lineage-tracing experiments (Greco et al. 2009; Hsu et al. 2011). It appears that some of the bulge-derived cells along the ORS layer on their transit from the bulge to the matrix are spared from the subsequent destructive phase and home back to be re-cycled to form the new bulge and/or hair germ based on their proliferative status and position on the ORS trail (Hsu et al. 2011).

Molecular regulation of hair cycling has been under intensive study and most data are based on analysis of transgenic and knockout mouse models with affected genes expressed intrinsically in the hair follicle. Several studies have suggested that Wnt and Bmp signaling exert positive and negative regulation, respectively, in guidance of hair growth. (Blanpain and Fuchs, 2006). Modulation of the level of sustained canonical Wnt pathway in epithelium causes formation of postnatal ectopic hair follicles (Gat et al. 1998; Lo Celso et al. 2004; Lowry et al. 2005; Silva-Vargas et al. 2005) or leads to precocious entry into anagen (Lowry et al. 2005; van Mater et al. 2003). Further, *de novo* hair formation occuring

upon deep wounding in normal mice is inhibited in the absence of signals from β-catenin, as well (Ito et al. 2007). When epithelial β-catenin is progressively lost after embryonic hair germ formation the hair follicles enter first catagen but fail to regrow (Huelsken et al. 2001). Ablation of β-catenin from dermal papilla leads similar hair cycling defect, as well (Enshell-Seijffers et al. 2010). Conditional knockout of *Bmpr1a* leads to activation of otherwise silent stem cells, which show elevated levels of β-catenin and Lef1 upon inactived Bmp signaling and proliferate, resulting in loss of slow-cycling cells but not hair follicle stem cells although they lose their ability to make hair. Continuous Bmp signaling in the bulge on the contrary inhibits stem cell activation and promotes premature hair follicle differentiation (Kobielak et al. 2007). Epithelial overexpression of *Noggin* causes hypertrichosis and faster transition to anagen (Plikus et al. 2004) and more selective misexpression of this Bmp inhibitor in matrix cells after formation of primary and secondary hair follicles results in premature entry into first catagen and subsequent loss of guard hairs (Guha et al. 2004).

The maintenance of telogen and activation of new growth phase appears to be the result of the reciprocal signaling between epithelial bulge and secondary hair germ, and mesenchymal dermal papilla when they are in close contact to each other. (Sun et al. 1991; Blanpain and Fuchs, 2006). Late telogen hair follicles show Wnt signaling in secondary hair germ and expression of Bmp inhibitors in dermal papilla (Greco et al. 2009). The transcriptional profiling of bulge stem cells has revealed that several components of the Wnt/β-catenin and the Tgf-β/Bmp signaling pathways are upregulated in the bulge enabling the stem cells to transmit signals from these pathways (Blanpain and Fuchs, 2006). The bulge appears to be a largely quiescent Wnt-inhibited environment except at the onset of new hair cycle (DasGupta and Fuchs, 1999; Tumbar et al. 2004). The bulge stem cell maintenance requires β-catenin and its activation breaks the niche silence causing transition of stem cells into proliferating transit amplifying cells and generation of new hair (Enshell-Seijffers et al. 2010; Yang and Peng, 2010). Couple of years ago it was shown that a wave of extrinsic dermal Bmp signals, involving Bmp2 and 4, which strengthen the intrinsic Bmp signals, competing with intrinsic Wnt signaling, create so called refractory phase of resting hair follicles which are not competent to respond to regeneration promoting signals. Decreased Bmp activity due to activity of Bmp inhibitors like Noggin enables telogen to transit into a competent phase allowing intrinsic Wnt signaling to activate bulge and hair re-growth. (Plikus et al. 2008).

Although studies have shown that the cycling is mainly a regulatory playground for Wnt and Bmp signaling, other modulators are known to be involved, as well. Shh has shown anagen inducing properties and growth promoting signals are thought arise from Fgf signaling, as well, probably through Fgf7/10 and Fgfr2IIIb signaling (Enshell-Seijffers et al. 2010; Greco et al. 2009; Petiot et al. 2003; Sato et al. 1999). The fur of *Fgf5*-null mice show abnormally long hair revealing similar phenotype to that of Angora mice with spontaneous mutation and implying that Fgf5 plays a role in negatively regulating hair elongation (Hébert et al. 1994). In dogs, Fgf5 has also been observed to affect coat pattern and hair length (Cadieu et al. 2009). In mice, Msx2 and Sgk3 appear to maintain the growth phase, and Tgf-β1 regulates the transition to catagen (Alonso and Fuchs, 2006). Interesting signaling loop for regulating telogen maintenance and anagen entry has been shown to include Hairless (Hr) and Wnt, and was suggested to involve Sostdc1. *Hr* mice are natural mutants characterised by hair loss and it has been suggested that Hr, which regulates gene transcription in ORS cells, represses *Sostdc1* expression at anagen onset to allow Wnt activation (Beaudoin III et

al. 2005). A very recent study proposes inducing signals from the neighboring adipocytes leading to bulge activation upon receving Pdgfa (Festa et al. 2011). Overall, it appears that the hair follicle coordinates both intrinsic and extrinsic signals to regulate its cycling (Jahoda and Christiano, 2011).

Table 1. *Selected murine genes affected in different signaling pathways showing embryonic and/ or postnatal hair formation abnormalities. HF=hair follicle; MVF=mystacial vibrissae follicle; HS=hair shaft; LOF=loss of function; GOF=gain of function; K14=keratin14 (expressed in skin basal cells); Cre=Cre-recombinase.*

Mouse model	Type of gene modulation	Pelage hair phenotype	Mystacial vibrissa phenotype	References
Lef1-/-	LOF	Reduced HF number	Loss of placodes	van Genderen et al. 1994
K14-Lef1	GOF	Postnatal ectopic HFs; mispatterning and misangling of HFs	Misangled, curved HSs	Zhou et al. 1995
K14-ΔN87β–cat	GOF	Postnatal ectopic HFs; hair tumors	Not described	Gat et al. 1998
K14-Cre (neo); β-cat $^{lox/null}$	LOF (Cre-mediated)	Loss of hair placodes	Not described	Huelsken et al. 2001
K14-Cre (Δneo); β-cat $^{lox/null}$	LOF (Cre-mediated)	Hair loss after first catagen	Not described	Huelsken et al. 2001
K14-Dkk1	GOF	Loss of placodes	Loss of placodes	Andl. et al. 2002
K14-Apc cko/cko	LOF	ectopic and misspatterned HFs; aberrant HS growth	Aberrant HS growth	Kuraguchi et al. 2006
Lgr4-/-	LOF	Reduced primary HF number	Not described	Mohri et al. 2008; Carmon et al. 2011
cytokeratin IV-Bmp4	GOF	Aberrant HFs; progressive HS loss	Aberrant MVs; loss HSs	Blessing et al. 1993
K14-Cre; Bmpr1A $^{flox/flox}$	LOF (Cre-mediated)	Lack of HSs	Lack of HSs	Kobielak et al. 2003
msx1-/-; msx2-/- (double knockout)	LOF	Reduced HF number	Not described	Satokata et al. 2000

Noggin-/-	LOF	Loss of secondary hair development	Not described	Botchkarev et al. 1999; Botchkarev et al. 2002b
Tgf-β2-/-	LOF	Reduced HF number; retarded moprphogenesis	Not described	Foitzik et al. 1999
ActivinβA-/-	LOF	Disturbed morphogenesis	Defective follicle differentiation	Matzuk et al. 1995 Nakamura et al. 2003
K14-Follistatin	GOF	Disturbed morphogenesis	Defective follicle differentiation	Nakamura et al. 2003
Fgf10-/-	LOF	Not observed	Reduced MV number and disorganized MV structure	Suzuki et al. 2000; Ohuchi et al. 2003
Fgfr2-IIIb-/-	LOF	Reduced HF number; retarded growth	Reduced MV number	Petiot et al. 2003
K5-Cre; Fgfr1/ Fgfr2	LOF (Cre-mediated)	Normal HF morphogenesis but progressive HS lost	Loss of HS	Yang et al. 2010
Shh-/- or Gli2-/-	LOF	Developmental arrest at bud stage	Reduced MV number	St.Jaques et al. 1998; Chiang et al. 1999; Mill et al. 2003
Gli3-/-	LOF	Not observed	Supernumerary MV	Mill et al. 2003
tabby/ downless/ crinkled	LOF	Loss of primary hair development; loss of auchenes and zigzags	Normal development	Headon et al. 2001; Monreal et al. 1999; Srivastava et al. 1997 Cui et al. 2010
K14-Eda	GOF	Enlarged hair placodes; prolonged anagen; curly HSs	Curly HSs	Mustonen et al. 2003; Mustonen et al. 2004
K14-Cre; Notch1 $^{lox/lox}$	LOF (Cre-mediated)	Premature entry into first catagen; dramatic reduction in postnatal HF number; abnormal HS structure	Not described	Vauclair et al. 2005

1.4. Mammary gland development

Mammary gland development is initiated during embryogenesis resulting in determination of nipple sheath and formation of mammary ductal anlage. The postnatal development involves intensive branching to form the mature glandular structure. The following represents the key events during embryonic and postnatal mammary morphogenesis, molecular regulation of the development, and nipple structure and formation.

1.4.1 Mammary gland

The first amniotes, terrestrial vertebrates protected their eggs from dehydration by moisturizing them with liquids secreted from their glandular skin. The present mammary gland has most probably arisen through further development of this glandular tissue requiring clustering of glands to specific regions and evolving to function only as a source of nutritions by enriching the nutritional value of secreted milk. Before acquiring nipple for targeted delivery of milk, the glands were associated with an areola covered with hairs which probably served both as feeding mechanism for the hatchlings and helped in liquid distribution over eggs. The present form, a hairless areola with nipple, requires less effort from the young to suck milk (Widelitz et al. 2007). The number of mammary gland pairs varies from one in humans to nine in pigs. Mice have five glands: three thoracic and two inguinal (see figure 9). There appears to be a correlation between the number of young and mammary glands (Schultz, 1948).

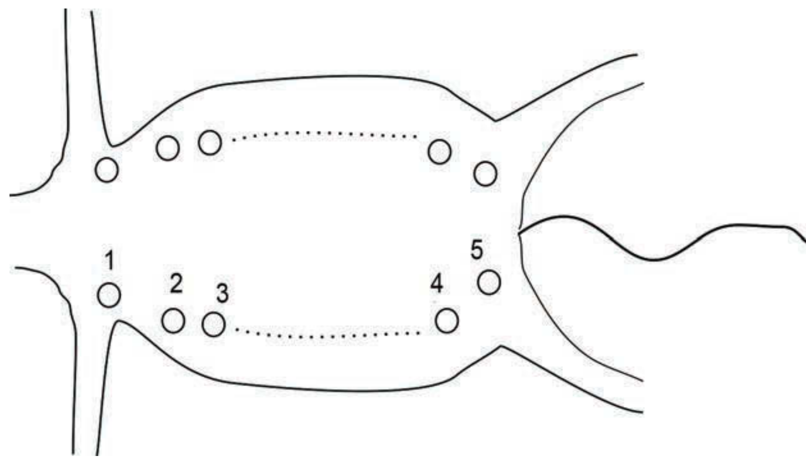

Figure 9. Murine patterning of mammary glands.

Ventral view of murine skin showing five pairs of mammary glands formed along the presumptive paralle milk lines (dotted line) running in anterior-posterior direction. Numbers 1–3 form the thoracic and 4 and 5 the inguinal pairs.

Mammary gland development can be devided into three stages: embryonic, pubertal, and adult. Compared to several other organs, the developmental processes of mammary gland occur mainly postnatally, and reproductive hormones play major part in controlling the development especially during puberty and pregnancy. The early embryonic development, on the other hand, appears to dependent solely on the reciprocal epithelial-mesenchymal signaling mediated by the conserved signaling pathways. Later, both during embryonic and postnatal development, the hormonal signals from the surrounding environment need to be transduced through interfering with the short-range epithelial-mesenchymal signaling. Some of these developmental regulators are also implicated in breast cancer (Robinson et al. 1999; Howard and Ashworth, 2006).

1.4.2 From milk line to mammary gland rudiment

The embryonic mammary gland morphogenesis is depicted in the figure 10**.** Mouse mammary development begins in both males and females by the formation of the two lateral milk lines around E10.25-10.5 running in anterior to posterior direction and located laterally to the ventral midline and between the developing fore- and hindlimbs (Cowin and Wysolmerski, 2010). Rats, rabbits, and humans show milk line as an ectodermal ridge but the existence of mouse mammary line as an anatomical structure is controversial and is rather identified by low-level expression several Wnt ligands, like *Wnt10b* and *Wnt6,* and canonical Wnt reporter gene *TOP-gal* and appearing first both in mesenchyme and epithelium (Chu et al. 2004; Veltmaat et al. 2003; Veltmaat et al. 2004).

The earliest promoting cues for mammary line positioning is believed to arise from *Fgf10* expressed by dermomyotome of the somites lying close to the milk line and the forelimb bud. Further, the Hh pathway component, Gli3, and Pax3 appear to be required for the somitic *Fgf10* gradient formation (Veltmaat et al. 2006). The presumptive receptor for Fgf10 is Fgfr2IIIb but its mRNA has not been detected in the early milk line. *Fgfr2IIIb* shows ectodermal expression around E11.5 in the mammary epithelium and becomes co-expressed in the placodes together with *Fgfr1* (Mailleux et al. 2002). Proposed Gli3-mediated Fgf10-Fgfr2IIIb signaling leads to downstream activation of Wnt pathway, by inducing mesenchymal/epithelial *TOP-gal* and epithelial *Wnt10b* expression in the mammary line (Veltmaat et al. 2006). Other early Wnt ligands that have been observed are *Wnts 3* and *6* showing broad band in flank ectoderm and *Wnts 5a* and *11* in the mesenchyme (Chu et al. 2004). Wnt signaling appears to be required for mammary induction as overexpression of Dkk1 in skin epithelium blocks *TOP-gal* and *Wnt10b* expression in the presumptive mammary line and appearance of all the five placode pairs but does not affect *Fgf10* expression (Chu et al. 2004; Veltmaat et al. 2004). Furthermore, tissue culture studies with induced Wnt signaling by Wnt3a or lithium chloride resulted into formation of enlarged mammary placodes (Chu et al. 2004).

Transcription factor T-box (Tbx) 3, which has been implicated in human mammary-ulnar syndrome and detected in the E10.25 milk line, is thought to first act downstream of Fgf and Wnt signals but then amplifies these signals by inducing certain Fgf and Wnt pathway components, like *Wnt10b* , in the mammary line and additionally *Lef1* later in the placode (Davenport et al. 2003; Eblaghie et al. 2004; Hens and Wysolmerski, 2005). Signals from the mesenchymal neuregulin (Nrg) 3, which is expressed as early as E10.75 prior to placode

appearance, mediated through its cognate RTK Erbb4, has been suggested to augment the *Tbx3* expression in the placode epithelium and acting upstream of Wnt pathway in placode induction (Howard et al. 2005; Robinson, 2007). Positive signals are also provided by Eda/ Edar signaling as transgenic *Eda-A1* overexpressing mice reveal ectopic mammary placodes around E12 which give rise to mammary glands along the postnatal mammary line. Eda signaling is not, however, crucial for mammary gland induction as *Eda*-deficient embryos show all mammary placodes (Mustonen et al. 2003; Mustonen et al. 2004).

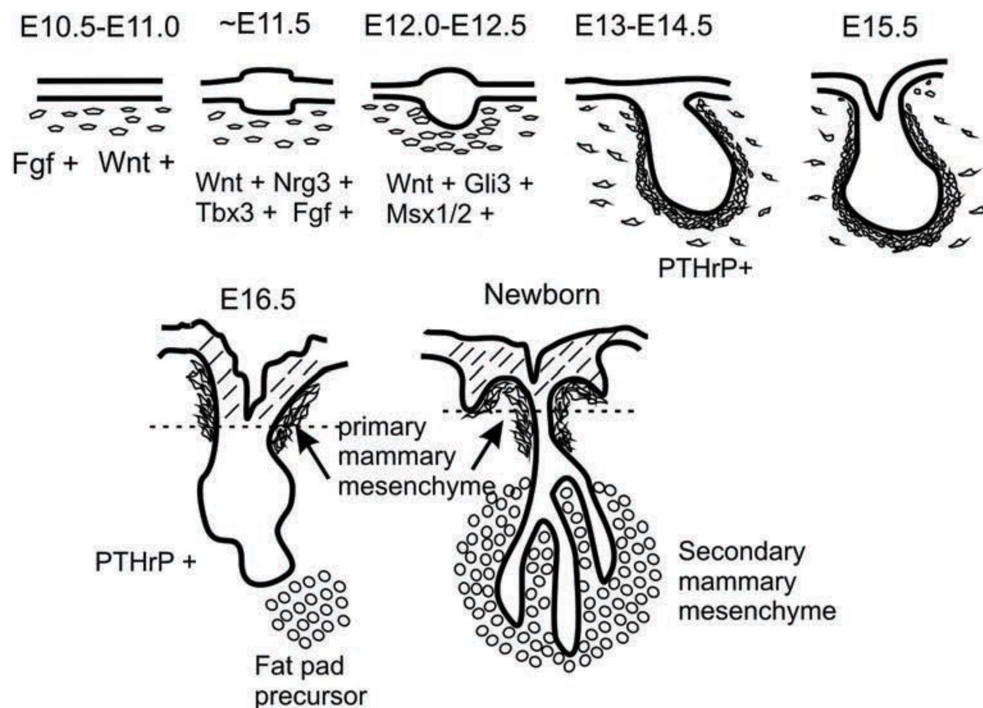

Figure 10. Embryonic mammary gland morphogenesis.

The mammary gland development is thought to begin by formation of a milk line, showing Tbx3 expression and Wnt activity, which defines the region for the arising mammary placodes around E11.5. First steps of development are regulated by Fgf, Wnt, Nrg3, Gli3, and Tbx3. By E12.5 the placode invaginates to form a bud, which is guided by Wnt signaling, Gli3, and Msx1/2. Bud grows further downward. Pthrp signaling induces the appearance of the concentric layers of fibroblasts around the bud and thus, a condensed primary mammary mesenchyme is formed and signals from it are required for nipple sheath specification around E16.5. The mammary bud remains rather silent during E13-E15.5 increasing only sligthly in bud size. Around E16 the proximal tip of mammary bud begins to sprout probably in response to Pthrp and Bmp4 signals. The primary mammary sprout invaginates to reach the borders of fat pad precursor after which it begins bifurcate. First branches dig into the fat pad, which will form the secondary mammary mesenchyme (stroma) to regulate postnatal ductal branching involving signals also from systemic factors.

According to *in vitro* studies, the dorso-ventral positioning of placodes has been suggested to be result of Bmp-Tbx3 interplay probably by defining the *Lef1* expression region to stimulate placode formation (Cho et al. 2006). The five lens-shaped multilayered mammary placodes (anlage) elevate above ectodermal surface and arise in dynamic and asynchronous fashion within ~24 hours showing an order where placode pair number 3 appears first, followed by 4 and then 1 and 5, and finally 2 (Veltmaat et al. 2004). Number 1 and 5 form ventrally to forelimb and hindlimb, respectively, at the border of limb and trunk.

The expression patterns of the early mammary line marker genes change from continuous line to spot-like pattern at the sites of placode formation but still low-level expression of these markers is detected between mammary anlage. The cellular mechanisms behind mammary gland formation are not well known. Studies have shown low levels of BrdU incorporation in the region of the developing mammary rudiments (Balinsky 1950; Lee et al. 2011). Further studies have suggested that cell migration instead of proliferation is thought to serve as a mechanism for the early mammary anlage formation but formal evidence for this is still missing (Balinsky, 1950; Propper, 1978; Lee et al. 2011; Veltmaat et al. 2003).

The appearance of the 10 mammary anlagen appear to rely on actions by the Wnt pathway and the transcriptional regulator p63, the latter also causing absence of other skin appendages in addition to mammary glands upon loss of its function (Andl et al. 2002; Yang et al. 1999). Analyses of several knockout mouse lines, however, have indicated differencies in the required signals for forming certain placode or bud pairs (see table 2). Deletion of *Lef1, Fgf10,* or *Fgfr2IIIb* spares only mammary gland 4, and the development of number 2 is often left unaffected upon loss of *Tbx3* (Davenport et al. 2003; Mailleux et al. 2002; van Genderen et al. 1994). The placode pair 3 requires signals from *Nrg3* and the formation of the bud pairs 3 and 5 need the repressive function of Gli3 (Hatsell and Cowin, 2006; Howard and Gusterson, 2000). Hypomorphic *Nrg3* induces ectopic mammary placodes around number 4, as well (Howard et al. 2005; Panchal et al. 2007). Epithelially targeted conditional ablation of transcription factor *Gata3* leads to variable loss of placodes (Asselin-Labat et al. 2007).

Similar to hair development, also mammary gland induction depends on Wnt and Fgf signaling but one remarkable difference is in the requirement of Hh pathway activity. Studies have shown that the developing mammary glands are devoid of *Gli1*- and *Ptc1*-lacZ reporter expression. Further, loss of *Shh*, *Ihh*, *Gli1*, or *Gli2* results in normal mammary gland development but silencing of Hh signaling by the repressor function of Gli3 is essential to mammary bud formation (Michno et al. 2003; Hatsell and Cowin, 2006) . In contrast, active Shh pathway is required for hair bud downgrowth (Chiang et al. 1999; St. Jaques et al. 1998). Moreover, it appears that Hh signaling plays a role in maintaining the hair identity of epithelial cells, as K14-Cre mediated deletion of *Smo* results not only to the loss of some hair follicles but also to the transformation of hair follicles to obtain more mammary gland-like features (Gritli-Linde et al. 2007).

The mammary placodes bud to the underlying mesenchyme by E12.5 but are still observed as knobs on the surface ectoderm. The process requires changes in cell adhesion and growth promoting signals from the Wnt pathway and regulation by Msx1, Msx2, and Gli3 (Satokata et al. 2000; Veltmaat et al. 2006). Further, ablation of *Lrp6* or *Lrp5*, whose protein products mediate canonical Wnt signals, leads to the formation of small E12.5 buds with reduced Wnt reporter *BAT-gal* expression (Lindvall et al. 2006; Lindvall et al. 2009).

Essential signals from the early bud by parathyroid hormone-related protein (Pthrp), which is an important regulator of bone remodelling, through its mesenchymal parathyroid hormone receptor (Pthr) 1, are required for the formation of the primary mammary mesenchyme, which begins to express the receptors for oestrogens and androgens, and the matrix protein tenascin C (Foley et al. 2001; Heuberger et al. 1982; Robinson et al. 1999; Wysolmerski et al. 1998). The mesenchymal fibroblasts form concentric layers around the invaginating bud to maintain mammary epithelial cell identity and to promote the mammary tree and nipple formation (the latter described in detail below; Robinson, 2007). In males, it responses to testosterone to induce destruction of the mammary bud around E14. This process is inhibited upon loss of *Pthrp* or *Pthr1*. (Dunbar et al. 1999).

The mammary bud grows in size during E12.5-E15.5 after which the bud tip grows down from the primary mammary mesenchyme and contacts the fat pad precursor. In males, the mammary bud gradually disappears, but in females, the primary sprout branches in dichothomous fashion into the fat pad, which becomes the secondary mammary mesenchyme (mammary fat pad), to form a rudimentary ductal network.From birth until puberty the development of the rudimentary mammary tree is largely silent. The molecular regulation of the embryonic mammary tree formation is not well known (Cowin and Wysolmerski, 2010). Loss of *Pthrp* blocks mammary development at late bud stage but the ductal outgrowth is rescued in culture conditions by addition of Bmp4 (Hens et al. 2007). Further, *Lrp6* shows expression both in mammary epithelium and fat pad, and *Lrp6*-null mice reveal underdeveloped mammary glands and fat pads prior to birth. These data suggest a role for Wnt pathway played also in regulation of the early ductal branching (Lindvall et al. 2009). Moreover, mice lacking *Pygo2*, which is a nuclear factor involved in Wnt pathway regulation, show similar mammary gland phenotype as *Lrp6*-knockouts (Gu et al. 2009; Kramps et al. 2002).

1.4.3 Postnatal mammary branching morphogenesis

At birth, the size and branching pattern varies in individual mammary trees, even between glands of a single pair and littermate pups. Thus, extrinsic factors probably play an additional role in ductal branching (Veltmaat et al. 2003). The perinatal mammary tree consists of 10-15 branches and the further growth of the ductal network is proportional to the body growth until puberty. The ductal outer layer is formed by myoepithelial cells which are underlined by layer of luminal cells. Adipocytes interspersed with fibroblasts form stroma surrounding the prepubertal mammary tree. The myoepithelial cells produce basement membrane components which cover the ductal system branches and form the direct contact to the stromal cells. (Watson and Khaled, 2008).

At the onset of murine puberty around three weeks of age, rising estrogen levels cause increased mammary gland branching and eventually the entire fat pad is colonized by the mammary tree (see figure 11; Richert et al. 2000). Estrogen is thought to induce ductal outgrowth by signaling through estrogen receptor α causing release of paracrine signals involving amphiregulin which is activated by a disintegrin and metallopeptidase domain 17 and activates the neighboring cells to proliferate (LaMarca and Rosen, 2007; Ciarloni et al. 2007; Korach et al. 1996; Mallepell et al. 2006; Sternlicht et al. 2005). This probably involves signaling through stromal Egf receptor, which is known to be essential for ductal

morphogenesis (LaMarca and Rosen, 2007; Wiesen et al. 1999). Further, studies have suggested that Gata3 through Foxa1 may be involved in promoting expression of estrogen receptor α or in the commitment to the estrogen receptor α-positive lineage (Watson and Khaled, 2008).

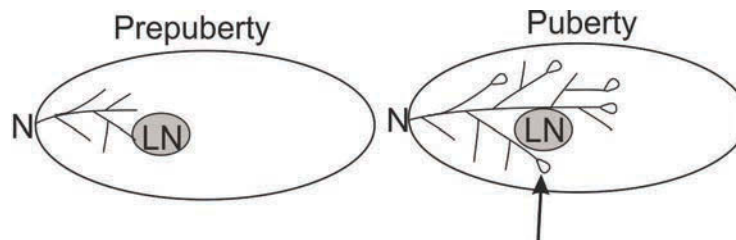

Figure 11. Development of mammary ductal tree in virgin mice.

A schematic model of mammary branching prior to and during puberty. At birth, mammary tree anlage consists of 10-15 branches and the branching is nearly inactive during the first three weeks of postnatal life. At puberty onset, the ducts elongate and bifurcate to reach the borders of mammary fat pad travelling further away from the nipple (N) and passing the lymph node (LN). Arrow indicates a TEB, which is considered to harbor mammary progenitor cells which form the myoepithelial and luminal cells of the mammary ductal walls.

The distally located growth-centers, terminal end-buds (TEBs), appear at the onset of puberty and form the functional structures responsible for ductal morphogenesis and show high level of proliferation in their cap cells which form both the luminal and myoepithelial cells (Watson and Khaled, 2008). The maintenance of luminal cells and TEBs appear to require Fgfr2IIIb signaling and deletion of this receptor gene causes defective pubertal branching (Parsa et al. 2008). TEBs meet the borders of the fat pad around 10-12 weeks of age and regress to form terminal ducts. In order to form lumen, cells die by apoptosis in the center of ducts (Humpreys et al. 1996). TEBs are considered to be rich in mammary stem cells and their activity is presumably maintained by Wnt signaling through Lrp5 (Badders et al. 2009; Lindvall et al. 2006). *Lrp5-/-* mice reveal reduced number of TEBs resulting in defective juvenile ductal branching and delay in Wnt1-induced mammary tumor formation (Lindvall et al. 2006). Further, heterozygous *Lrp6*-knockout mice show decreased number of TEBs by five weeks of postnatal development which eventually results in slightly reduced branching complexity of 11-week-old mammary trees but in *Lrp5-/-* background, *Lrp6+/-* mice show absence of all mammary trees although nipples are present (Lindvall et al. 2009).

Expression of *Wnt1* or *Wnt10b* under mouse mammary tumor virus (MMTV) promoter in luminal epithelia leads to hyperbranching of ductal tree. In MMTV-*Wnt10b* mice, precocious alveologenesis is observed, as well. Stabilized β-catenin results in similar branching phenotype but also in the formation of adenocarcinoma. (Incassati et al. 2010).

Wnt signaling is also involved in the negative regulation of pubertal ductal elongation and lateral branching as loss of *Wnt5a* accelerates ductal morphogenesis. Expression of *Wnt5a* was shown to be induced by Tgf-β signals (Roarty and Serra, 2007).

During pregnancy, endocrine factors, like hormone progesterone signaling through its receptor (progesterone receptor), induce lobuloalveolar precursor cells to colonize all the interductal regions, thus cell number increases by the massive proliferation approximately 10-fold (Henninghausen and Robinson, 1998). A family member of Tnf, receptor activator of Nf-κb-ligand (Rankl), appears to act downstream of progesterone as loss of this paracrine factor in the mammary epithelium abolishes progesterone induced morphogenesis (Beleut et al. 2010). Further, progesterone has been shown to induce Wnt4 in the mammary epithelium and loss of this paracrine factor results in poor ductal side-branching during early pregnancy, thus, likely involved in mediating the functions of progesterone (Boras-Granic and Wysolmerski, 2008).

In addition to progesterone, alveoli differentiation requires prolactin, and Wnt signaling (Watson and Khaled, 2008) and the process involves cleavage of alveolar buds to give to rise to individual alveoli. Mature alveoli begin to secrete milk upon parturition and lactation may last approximately three weeks. At weaning, the gland begins the returing to quiescent stage by tissue-remodeling involving apoptosis of mammary epithelial cells (Richert et al. 2000; Watson, 2006). Involution lasts around two weeks and during this time the mammary tree is capable of initiating another cycle of prenancy, lactation, and involution (Richert et al. 2000).

1.4.4 Mammary nipples

In mouse, the nipple epidermis is determined around E16.5 in response to signals from primary mammary mesenchyme, and studies have implicated Pthrp-Ptr1 signaling essential for nipple formation (Foley et al. 2001; Robinson, 2007). Loss of *Pthrp* or its receptor leads to absence of nipples and trangenic *Pthrp*-mice show in entire ventral skin expansion of primary mammary mesenchyme and formation of diffuse nipple-like epithelium with nipple specific keratins, thickened epidermis, and inhibition of hair follicle formation (Foley et al. 2001; Mahler et al. 2004). Otherwise, the molecular regulation of the nipple formation is poorly known. The epithelium overlying the mammary cord, later the lactiferous duct, invaginates in circular fashion (showing umbrella-like appearance), thus forming a nipple sheath by E18.5. The center of the nipple sheath rises outward forming the nipple anlage. In murine males, the testosterone is responsible for the absence of nipples. (Velmaat et al. 2003). The nipples become easily distinguishable macroscopically after puberty**.** To endure the mechanical strain of nursing, the further nipple development requires thickening of the epidermis by increasing suprabasal layers and expression of nipple specific keratins, like keratin-2e, (Mahler et al. 2004). The composition of keratins changes and the size of the nipple increases upon pregnancy (Eastwood et al. 2007). The nipple associated mesenchyme, known as connective tissue, differs from the surrounding skin mesenchyme by showing reduced organization of collagen bundles, and increased number of smooth muscle α-actin, mast cells, elastic fibers, blood capillaries, nerve fibers, melanocytes, and acidic mucopolysaccharides (Abdalkhani et al. 2002).

Table 2. *Selected mouse models showing embryonic mammary gland phenotype. MP=mammary placode; MB=mammary bud; E=epithelium; M=mesenchyme*

Mouse model	Type of gene modulation	Phenotype	Reference
Lef1-/-	LOF	Loss of MP2 and 3; developmental arrest at early bud stage of MB1 and 5	van Genderen et al. 1994
K14-Dkk1	GOF	Loss of all MPs	Chu et al. 2004
Lrp5-/-	LOF	Decreased MB size	Lindvall et al. 2009
Lrp6-/-	LOF	Decreased MB size	Lindvall et al. 2009
Fgf10-/-	LOF	Loss of MP1-3, 5	Mailleux et al. 2002
Fgfr2-IIIb-/-	LOF	Loss of MP1-3, 5and arrest of MB4 development	Mailleux et al. 2002
Gli3$^{xt/xt}$	LOF	Loss of MB3 and 5	Hatsell and Cowin, 2006
Tbx3-/-	LOF	Loss of MP1 and 3-5, MB2 present occasionally	Davenport et al. 2003
Ska (Nrg3)	LOF	Loss of MP3; ectopic placodes around MB4	Howard and Gusterson, 2000; Howard et al. 2005
K14-Nrg3	GOF	Ectopic placodes	Panchal et al. 2007
Msx2-/-	LOF	Developmental arrest at mammary sprout stage	Satokata et al. 2000
Msx1-/-; Msx2-/-	LOF	Developmental arrest at MP stage	Satokata et al. 2000
K14-Cre; Gata3 $^{flox/flox}$	Cre-mediated LOF	variable loss of MPs	Asselin-Labat et al. 2007
Pthrp-/- or Ptr1-/-	LOF	Developmental arrest at late MB stage	Foley et al. 2001
K14-EdaA1	GOF	Ectopic MPs/MBs along mammary line	Mustonen et al. 2004
p63-/-	LOF	Absence of all MPs	Yang et al. 1999; Laurikkala et al. 2006

2 AIMS OF THE STUDY

This work was based on three mouse models with modified Wnt signaling activity. The study focused on the development and patterning of hair and mammary gland. The main aims were:

1. To analyze the effects of sustained Wnt/β-catenin signaling in skin epithelium on hair development and study the downstream targets of the canonical Wnt pathway in this context.

2. To examine the role of the Bmp and Wnt antagonist, Sostdc1, in embryonic and postnatal hair and mammary gland development.

3. To study which pathway, Wnt or Bmp, Sostdc1 primarily regulates during different developmental stages of hair and mammary gland formation.

4. To analyze of the effects of the presumable Wnt and Eda target gene, *Fgf20*, on primary hair placode patterning.

3 MATERIALS AND METHODS

3.1 Mouse strains

Mouse strain	Used in article	Purpose
NMRI	I, II	mRNA expression studies, in vitro cultures
C57BL/6	II	mRNA expression studies, control strain for Sostdc1-/- mice
β–catenin$^{\Delta ex3K14/+}$	I	Activation of β-catenin in skin epithelium for hair phenotype analysis
Rosa26 reporter	II	LacZ reporter analysis
TOP-gal reporter	II	Analysis of Wnt/β–catenin signaling activity
BAT-gal reporter	I, II	Analysis of Wnt/β–catenin signaling activity
Sostdc1-/- (in C57BL/6 background)	II	*Sostdc1-/-* skin appendage phenotype analysis
K17-GFP	II	GFP expression analysis in *Sostdc1*-null skin appendages
Tabby (B6CBACa-A^{w-J}/A Ta/0 Jackson Laboratory, Bar Harbor, USA)	I	Hair phenotype analysis of β–*catenin*$^{\Delta ex3K14/+}$ mice in *tabby* background
Fgf20$^{lacZ/lacZ}$	unpublished results	Embryonic hair phenotype analysis of *Fgf20*-knockout mice
Nude	I	Skin transplantation

Out of the several available Wnt reporter mice, two were chosen for this thesis work: TOP-gal (DasGupta et al. 1999) and BAT-gal reporter mice (Maretto et al. 2003). Both reporter lines express *beta-galactosidase* (or *LacZ*) gene under control of b-catenin TCF responsive elements upon Wnt/β-catenin activation. In TOP-gal mice, three LEF/TCF binding sites are fused to c-fos minimal promoter and in BAT-gal reporter line, seven LEF/TCF bindins sites are fused to minimal promoter - TATA-box of the *Siamois* gene.

2–10 mutant mice were used for tissue culture or histological and expression analysis unless otherwise stated.

The appearance of a vaginal plug was taken as embryonic day 0. The precise embryonic day/developmental stage of each individual within a litter was further defined by morphological criteria based on limb development (E10.5 onwards) and number of vibrissae (E12–E14).

3.2 Probes for in situ hybridisation

Probe	Used in article	Reference
Sostdc1	I, II, unpublished results	Laurikkala et al. 2003
Bmp4	I, unpublished results	Vainio et al. 1993; Mustonen et al. 2004
Bmp2	I	Pispa et al. 1999
Bmp7	unpublished results	Åberg et al. 1997
Wnt10b	I, II	Wang and Shackleforck, 1996
β-catenin	II	Laurikkala et al. 2002
Lef1	II	Travis et al. 1991
Dkk1	I	Andl et al. 2002
Shh	I	Vaahtokari et al. 1996
Edar	I	Laurikkala et al. 2001
Id1	II	Rice et al. 2000

3.3 Antibodies used in the study

Antibody	Host	Source/Reference	Used in article
Keratin2e	mouse	Fitzgerald	II
Ki-67	rabbit	Neomarkers	II
BrdU	mouse	Neomarkers	II
Sox2	rabbit	Millipore	unpublished results
P-cadherin	rat	Devenport and Fuchs, 2008	unpublished results
Lef1	rabbit	Cell Signaling Technology	II
Phospho-SMAD1/5/8	rabbit	Cell Signaling Technology	I, II
EpCAM	rat	BD	II
Keratin82	rabbit	Abgent	II

Sodium citrate-treated (10mM, pH 6.0) paraffin sections were used to detect Sox2 and P-cadherin, using anti-Sox2 (1:2000) and P-cadherin (1:100) with proper secondary fluorescent antibodies (Alexa Fluor).

3.4 Methods

Method	Used in article
Histology	I, II
Tissue culture	I, II
Whole-mount in situ hybridisation	I, II
Radioactive in situ hybridisation on sections	I, II
Immunohistochemistry	I, II
X-gal staining	I, II
Scanning electron microscopy	I
Mammary gland staining	II
Quantitative RT-PCR	I
Hair analysis	I, II
Sweat analysis	II
Skin transplantation	I
Oil Red O staining	II
Confocal microscopy	II
3D analysis of mammary development	II
Cell proliferation assay (BrdU)	II

4 RESULTS AND DISCUSSION

4.1 Dynamic expression pattern of *Sostdc1* during embryonic mammary gland and hair and vibrissal follicle development (I, II).

Before analysing the *in vivo* effects of deleted *Sostdc1* on mammary and hair development, we analyzed its embryonic expression pattern. Earlier it had been shown, that *Sostdc1* is localized to E12 skin ectoderm, in vibrissal placodes, buds, and the IRS of emryonic follicles, and E14 pelage hair placodes (Laurikkala et al. 2003). We observed prominent ectodermal localization in E11 nascent vibrissa pads prior to placode appearance and later, *Sostdc1* transcripts were epithelially confined to the immediate surroundings of the pelage hair placodes and primary and secondary vibrissal placodes/early buds, showing a ring-like expression pattern around these organ primordia. Further, *Sostdc1* showed an even distribution to the whole E13.5 vibrissa pad region filling the interfollicular spaces. By E15, *Sostdc1* had shifted from the surroundings of the hair germs to bud epithelium (unpublished results). As a comparison, also chick feather buds first show similar epithelial ring-like expression pattern of *Sostdc1*, which is later dynamically changed as the feather development proceeds but also mesenchymal expression was observed (Shigetani and Itasaki, 2007).

Interestingly, an earlier study of Sostdc1 protein expression during rat embryonic and postnatal hair development has shown localization in mesenchyme and basal lamina as well as in the epithelial compartments where we detected the mRNA expression. (O'Shaughnessy et al. 2004). Although our data revealed only epithelial localization of *Sostdc1* in embryonic snout and dorsal skin , the protein expression in mesenchyme could be explained by the fact that Sostdc1 is a secreted and soluble molecule presumably diffusing into the two tissue layers. Further expression analysis shown as unpublished data (see figure 12), however, revealed that in postnatal hair follicles, *Sostdc1* was expressed in dermal papilla but was also observed in the upper hair bulb likely in hair cortex and matrix compartments. *Sostdc1* was absent from telogen hair follicles. These findings are in consistent with earlier gene expression data where *Sostdc1* was shown to disappear during late catagen and reappear by late anagen in the hair bulb region (Beaudoin III et al. 2005).

The expression of *Sostdc1* in embryonic mammary glands has not been reported earlier. The earliest signs of *Sostdc1* transcripts were detected as a faint stripe around E11 in the region of future thoracic mammary placodes. More convincingly, the presence of *Sostdc1* was detected by E12.0 when mammary placodes 2 and 3 were observed with surrounding and diffuse *Sostdc1* expression. Sectional in situ hybridization confirmed the mesenchymal localization of *Sostdc1* under E12.0 mammary placodes from where it shifted to epidermal flanks of E13.5 mammary bud but was absent from the organ primordia itself. Mesenchymal expression was observed in the early mammary buds, as well, although it was less intensive compared to that of skin epithelium. Later, *Sostdc1* had shifted from the neighbouring tissue to inside of the mammary bud by E15.5 and at E17.5, it was observed in the mammary sprout

and lining the borders of the mammary bud epithelium. A similar dynamic expression pattern of *Sostdc1* has been reported earlier in embryonic mouse tooth development, showing first mesenchymal expression and then detected in both compartments (Laurikkala et al. 2003; Munne et al. 2009).

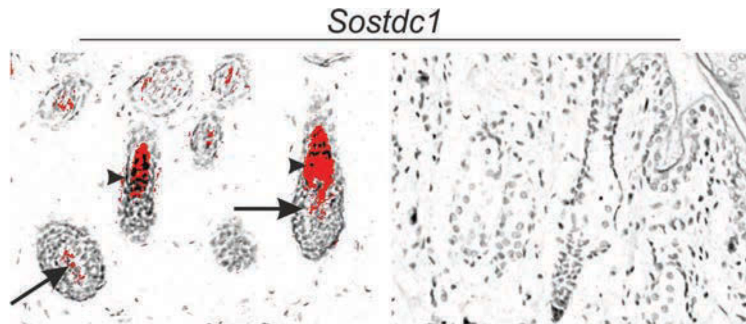

Figure 12. Sostdc1 expression in postnatal hair follicles.

Radioactive in situ hybridisation revealed that Sostdc1 is expressed in the dermal papilla (arrow) and upper region of epithelial hair bulb (arrowhead) probably in the matrix and the hair cortex. No expression of Sostdc1 was detected in telogen hair follicles presented on the right.

4.2 Hair and vibrissa placode induction and patterning is affected by modulation of Wnt signaling (I, II, unpublished results).

Earlier it has been shown that Wnt signaling is necessary for pelage and primary vibrissal hair placode initiation (Andl et al. 2002; Huelsken et al. 2001; vanGenderen et al. 1994). Furthermore, ectodermal overexpression of degradation-resistant β-catenin, resulting in forced activation of β-catenin mediated canonical Wnt signaling, causes ectopic feather buds in embryonic chicks and *de novo* formation of postnatal hair follicles but normal embryonic hair development in different β-*catenin* gain-of-function function mouse models (Noramly et al. 1999; Gat et al. 1998; Lo Celso et al. 2004; Silva-Vargas et al. 2005).

To re-examine the effects of sustained β-catenin activity on embryonic hair induction, we used *Catnb*$^{\Delta ex3K14/+}$ mice which are generated by crossing K14-Cre (Huelsken et al. 2001) with β–catenin-flox-exon3 mice (Harada et al. 1999). In these β-*catenin* transgenics, K14 promoter activation in skin basal cells from E11 onwards produces Cre recombinase to cut the exon3 flanked by loxP sites in β-*catenin.* Exon3 is responsible for producing the phosphorylation site required for degradation of β-catenin. Previously, similar mutant mice with stabilized β-catenin had shown supernumerary teeth and taste papillae during embryogenesis (Järvinen et al. 2006; Liu et al. 2007).

By analysing the expression of several hair placode marker genes, we were able to observe hair induction already at E12.5 on the shoulders of the forelimbs, overlapping the

site of Cre recombinase activity, and within a day, hair placodes covered largely the whole mutant body at a time when control mice showed pre-placodes on the ectodermal flanks. E14.0-E14.5 trunk and head skin revealed a dramatic appearance of placodes of variable sizes and in excessive amounts with lost regular patterning of hexagonal arrays showing a notably bumpy-like appearance on the skin surface. Placodes were detected everywhere in the skin, even in regions where hair follicles do normally never appear, like the ventral palms, or form later in development along for example the dorsal midline and tail skin. Even the inhibitory regions around the mammary buds, which otherwise showed largely unaffected embryonic development, or interfollicular areas in vibrissa pad region were disturbed by intrusive hair placodes. Further, the morphology of the affected skin showed both thickened epithelium and upper dermis.

Although the whole skin was able to obtain a hair fate, there were interfollicular spaces left, probably suggesting that Lef1/Tcf were still under regulatory control. Placode inhibitors, like Dkk1 and Sostdc1, showed upregulated expression levels but failed to suppress excessive placode formation. Further gene expression analysis by *in situ* hybridisation and quantitative PCR revealed increased levels of *Shh* which is known to act downstream of the Wnt pathway during embryonic hair development (Huelsken et al. 2001; Andl et al. 2002). Overexpression of *β-catenin* in adult skin causes upregulation of *Shh*, as well (Gat et al. 1998; Lo Celso et al. 2003; Silva-Vargas et al. 2005). Interestingly, we observed increased levels of *Bmp2* and *Bmp4*, the latter also showing ectopic localization in β-*catenin* mutant epithelium in addition to mesenchymal compartment at sites of placode formation. Further, phosphorylated Smad1, 5, and 8 showed enhanced localization in E14.5 skin epithelium and revealed continuous expression lines in mesenchyme. A similar epithelial and upper dermal expression pattern of *LaZ* was observed in BAT-gal reporter mice (Maretto et al. 2003; see Materials and methods) demonstrating Wnt activity. Thus, epithelially stabilized β–catenin results in increased epithelial and mesenchymal Wnt and Bmp signaling. Wnt/β-catenin signals may indirectly or directly regulate the Bmp pathway during hair induction and the interaction between these two pathways, Wnts promoting and Bmps inhibiting hair formation, may regulate proper hair placode patterning.

In addition to our experiments, three other independent studies were performed within a three year time frame to analyse the effects of continuous canonical Wnt signaling either by stabilizing β-catenin in *KRT14-Cre Ctnnb1*$^{(Ex3)fl/+}$ (Zhang et al. 2008) or *K5-Cre Catnb*$^{(ex3)fl/+}$ mice (Suzuki et al. 2009), or deleting *Apc* in *K14-Cre;Apc*$^{cko/cko}$ mice (Kuraguchi et al. 2006). Precocious hair induction, as analysed by early placode marker expression, was reported in both β-*catenin* mutants but not in mice lacking *Apc*. *KRT14-Cre Ctnnb1*$^{(Ex3)fl/+}$ mice showed the earliest hair induction of all the mouse models likely reflecting the timing of Cre expression (Zhang et al. 2008). The three β-catenin gain-of-function mice are the first known mouse models showing precocious hair induction. Similar to our β-*catenin* mutant, all the other three mouse models had excessive amounts of mispatterned hair placodes with ectopic formation in hairless region. However, in contrast to our mouse model *KRT14-Cre Ctnnb1*$^{(Ex3)fl/+}$ mice showed that the whole skin eventually adopted hair fate without any interplacodal spacings (Zhang et al. 2008) suggesting again earlier and/or stronger Cre expression. Further, upregulated expression of transcripts from Shh pathway components and Wnt inhibitors (e.g. Dkks) were also upregulated in the other two β-*catenin* mutants corroborating our results. Support for our observation that Bmp signaling is regulated by Wnt pathway was also given by the other two β–*catenin* mutants showing upregulation of

Bmp2 and *Bmp4*. Analysis of *K5-Cre Catnb*$^{(ex3)fl/+}$ mice further revealed enhanced pSmad1,5,8 levels and ectopic epithelial *Bmp4* at sites of mutant placode formation similar to our studies but also suggested that *Bmp4* is not a direct β-catenin target gene and rather that *Bmp2* is upregulated through Shh (Suzuki et al. 2009).

4.2.1 Wnt/β-catenin signals guide initiation of primary hair placode formation upstream of Eda/Edar pathway.

Similar to the Wnt/β-catenin pathway, Eda/Edar signalling also stimulates placode formation (Mustonen et al. 2003; Mustonen et al. 2004) and they are both considered as early regulators of hair development and their hierarchical order has been under study (Huelsken et al. 2001, Andl et al. 2002; Laurikkala et al. 2002; Fliniaux et al. 2008; Zhang et al. 2009). Earlier it was shown that a conditional loss of β-*catenin* in mice causes absence of all hair placodes but does not affect *Edar* expression suggesting that Wnt is downstream of Eda-Edar signals (Huelsken et al. 2001). The resulting eembryonic hair phenotype in K14-*EdaA1* transgenics is different to that observed in our *Catnb*$^{\Delta ex3K14/+}$ mice. Excess of *Eda-A1* is unable to induce ectopic or precocious hair development although fused and enlarged placodes have been observed (Mustonen et al. 2004).

We decided to cross the *Catnb*$^{\Delta ex3K14/+}$ mice with *Eda -/-* (*tabby*) mice to analyse the relationship of the two signaling pathways. The E13-E15 compound mutant embryos showed the dramatic hair phenotype detected in the *Catnb*$^{\Delta ex3K14/+}$ skin, thus increased Wnt/β-catenin signaling compensated for the lack of Eda signaling in the first wave hair placodes. This finding would implicate Eda/Edar signaling to act upstream of Wnt/β-catenin signaling. Further, although *Edar* was expressed in all the placodes of mutant skin, qPCR revealed unaltered epithelial *Edar* expression level in E14 *Catnb*$^{\Delta ex3K14/+}$ skin. Thus, although Edar has been shown to function independent of Eda if its expression is moderately enhanced (Mou et al. 2006), this mechanism unlikely explains our observations. Interestingly, in the other β-catenin mutant, *KRT14-Cre Ctnnb1*$^{(Ex3)fl/+}$, *Edar* (but not *Eda*) was upregulated in E15 skin (Zhang et al. 2008). The differences between these mouse models could be explained by different stage of examination or alternatively by the fact that we used whole skin whereas in the other study, separated epithelium was analysed to measure epithelial *Edar* levels.

A previous search for putative Eda target genes by micro-array screen using embryonic skin shortly exposed to recombinant Eda protein, revealed induced *Dkk4* and *Lrp4* expression but failed to show any obvious Wnt agonists (Fliniaux et al. 2008). By *in vitro* studies, the expression of these genes was suggested to depend on Wnt signaling which is active prior to the Eda pathway. By qPCR analysis, Eda was unable to induce the expression of β-*catenin* or *Lef1* in E14 skin explants (Fliniaux et al. 2008) but very recently we have observed that Eda is able to stimulate both *Wnt10b* and *10a* in E13 mammary buds (Voutilainen et al. 2012). We hypothesize that placode induction requires Wnt/β-catenin acting upstream of Eda/Edar pathway, but during placode formation both pathways may act in co-operative manner perhaps to stabilize early placodes by controlling common target gene expression or cellular mechanisms through different genes. In support of the hypothesis of interactive and co-operative function, a recent study suggested that Wnt and Eda/Edar signals are reciprocally needed to induce hair development (Zhang et al. 2009). In line with

earlier findings (Fliniaux et al., 2008), this study also showed with Wnt and NF-κB reporter mice that activation of Wnt signaling is an Eda/Edar independent in preplacodes but at later stages, Eda/Edar/NF-κB signals set-up and maintain the strict focal Wnt activity (Zhang et al. 2009). It was proposed that Edar regulates the expression of *Wnt10b* and possibly also *Wnt10a* in placodes which further supports Wnt signaling during later morphogenetic steps. Further, Eda/Edar/NF-κB activation appear to require canonical Wnt signaling, as it was shown that β-catenin directly induces ectodermal *Edar*. *In vivo* overstimulation of the Eda pathway by constitutively activated ligand-independent *Edar* failed to create patterned NF-κB signaling and rescue primary hair follicle formation in mice with defective Wnt/β-catenin signaling. (Zhang et al. 2009). These and our results support the hypothesis of canonical Wnt signals are upstream of all the other pathways during hair follicle induction.

4.2.2 Ablation of *Sostdc1* leads to enlarged primary hair placodes and increased vibrissa follicle number

The intriguing ring-like expression pattern of *Sostdc1* around hair placodes and its upregulation in $Catnb^{\Delta ex3K14/+}$ mice prompted us to study the hair placode phenotype of *Sostdc1*-null mice. The knockout mice showed no difference in hair follicle spacing; however, a slightly increased placodal area was observed as defined by the expression of *Wnt10b* or GFP expressed under the K17 promoter although the placode morphology was grossly normal. An additive effect on placode size was expected in compound *Sostdc1-/-;* $Catnb^{\Delta ex3K14/+}$ mice but the skin phenotype was similar to the E14 $Catnb^{\Delta ex3K14/+}$ (data not shown). β-catenin stabilization likely causes insensitiveness to all inhibitors upstream of β-catenin. The suggested interplay of the Eda and Wnt pathway in hair placode induction intrigued us to analyse compound E14 *Sostdc1*-/-;K14-*EdaA1* dorsal skin, as well, but loss of *Sostdc1* did not affect K14-*EdaA1* hair placode phenotype as studied by *Wnt10b* expression pattern (data not shown). Knowing that the placode patterning involves tight regulation of Wnt and Bmp induced signals by other significant regulators, such as Dkks and Noggin (Andl et al. 2002; Botchkarev et al. 1999; Sick et al. 2006), Sostdc1 appears to serve a less important role in primary hair placode patterning.

Surprisingly, the *Sostdc1*-null embryo showed supernumerary vibrissal placode formation in the mystacial vibrissa pad especially between rows 3 and 4, above the most dorsal row 5, and among developing post-orbital and inter-ramal sensory hairs, as examined by marker gene expression and tissue culture of vibrissa pad and mandibular skin showing GFP expression under the K17 promoter. These ectopic placodes appeared around E13.5 and eventually gave rise to proper hair filaments in adult mice, as well. Together these observations and the detected expression pattern of *Sostdc1* in the whole vibrissa pad region and around the developing follicles imply Sostdc1 to act as a regulator of correct vibrissa number, similar as in tooth formation.

The disturbed molecular mechanisms resulting in this vibrissa phenotype, however, were left without an answer. The ectopic vibrissal placodes showed expression of *TOP-gal* and Wnt pathway components but the overall Wnt activity (detected by TOP-gal; see Materials and methods) was not enhanced. Furthermore, introduction of Dkk1 or Noggin failed to prevent the appearance of extra mystacial vibrissae in three-day tissue cultures of E12.5 vibrissa pads (data not shown). Despite these observations, it would be tempting to think of Sostdc1 as a

primary Wnt inhibitor in this context, as Wnt signaling is thought to promote hair placodal fate and Bmp is considered to negatively regulate hair induction (Millar, 2002). The blocking of Wnt signaling leads to the absence of all hair type placodes, whereas the inhibition of Bmp activity by overexpression of noggin causes extra whisker filament formation that share the same orifice with the main vibrissae (Plikus et al. 2004). Further, although *Catnb*$^{\Delta ex3K14/+}$ mice showed unaffected initial vibrissal patterning, the vibrissae pad region revealed ectopic hair placodes and buds densely arising in the intervibrissal spaces. Similarly, extra postoral and inter-ramal vibrissae form in K14-*EdaA1* mice (M. L. Mikkola, unpublished data). The link between Sostdc1 and Eda could be mediated through the Wnt pathway as *Edar* has been suggested to be a Wnt target gene and Edar may induce the expression of Wnt ligands (Zhang et al. 2009).

In general, however, the molecular mechanisms governing precise vibrissa patterning or development are poorly known, although it is believed that similar mechanisms are used as in pelage hair formation. Studies have suggested that there are some variations in signals used for mystacial vibrissae versus pelage hair formation and these findings would also explain why we observe more changes in vibrissa phenotype than in that of pelage hairs of *Sostdc1*-null mice. *Fgf10* and *Lef1* appear to be more essential for mystacial vibrissae but their contribution can be totally (*Fgf10*) or partly (*Lef1*), substituted in induction of trunk hair follicles (van Genderen et al. 1994; Ohuchi et al. 2003). Further, loss of *Gli3*, or virally induced overexpression of Shh in facial skin prior to vibrissa placode appearance, leads to ectopic mystacial vibrissal formation (Mill et al. 2002; Ohsaki et al. 2002). Interestingly, although Shh signaling has not been thought to play a role in pelage hair follicle initiation (Chiang et al. 1999; Huelsken et al. 2001; St.Jaques et al. 1998) lack of its expression, suppresses the embryonic hair phenotype caused by stabilized β-catenin (Suzuki et al. 2009). Thus, Shh may function early on in hair formation. Using tooth explants, it has previously been shown that Shh-releasing beads inhibit the ability of Bmps to induce *Sostdc1* expression (Laurikkala et al. 2003), giving one possible explanation how Sostdc1 and Shh signaling could be linked during vibrissa development.

4.2.3 Wnt and Eda target gene, *Fgf20*, regulates dermal condensate formation during primary hair follicle formation

Microarray data, obtained from Eda-deficient skin samples treated with recombinant EDA-A1 discovered a possible new placode growth factor, *Fgf20* as Eda target gene (Fliniaux et al. 2008; Lefebvre et al. 2012). As another earlier study has implied that *Fgf20* is a direct Wnt target gene (Chamorro et al. 2005) we were intrigued to analyse the effects of ablated *Fgf20* on pelage primary hair placode formation and patterning using *Fgf20*-null mice (Huh et al. 2012). *Fgf20* showed epithelial expression in hair placodes (data not shown) and thus, we studied E14 knockout mice by expression analysis combined with histological examination (Figure 13). Histological analysis of mutant skin revealed the presence of morphologically rather normal placodes with *Shh* expression (Fig. 13A) and P-cadherin (Fig. 13B). Despite the normal appearance of *Fgf20*-null placodes, the skin surface appeared more flat compared to the slightly bumpy skin at the sites of hair formation in the control mice. The elevated placodes likely results from the mechanistic action of the dermal condensate, well known in feather bud formation. Interestingly, careful analysis of histological sections revealed

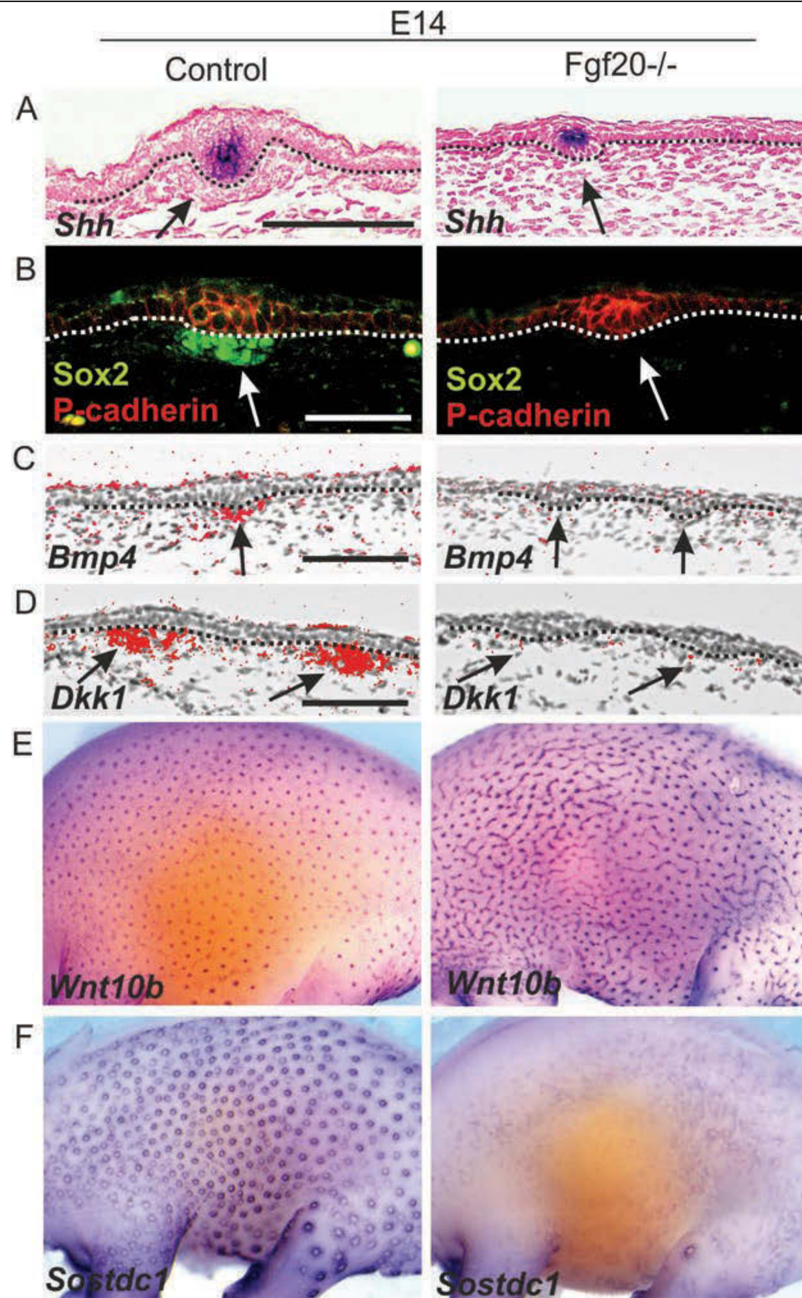

Figure 13. Ablation of Fgf20 leads to altered patterning of placodal gene expression and loss of dermal condensate markers in pelage primary hair placodes.

*(**A-F**) Analysis of mRNA (A, C-F) or protein (B) expression (B) of hair placode associated markers in E14 control and Fgf20-null mice. (A) Nuclear fast-red counterstained sections with Shh expression shown in hair placodes of whole-mount in situ hybridisation specimens. Histology of the Fgf20-null skin shows even surface of ectoderm and absence of visible dermal condensate (arrow), detected clearly in control section. (B-D) Detection of the mesenchymal condensate markers Sox2 by immunohistochemistry (B) and Bmp4 (C) and Dkk1 (D) by radioactive in situ hybridisation revealed expression of these markers in control dermal compartment of hair placodes but were absent from Fgf20-null mesenchyme. P-cadherin protein was observed in the epithelium of hair placodes both in the control and mutant skin (B). (E,F) Whole-mount in situ hybridisation revealing stripy and epithelial Wnt10b expression (E) and absence of Sostdc1 (F) around the hair placodes in Fgf20-null mice. Control mice show a spot-like expression pattern of all these placode markers. Arrows point to placodes. Scale bars 200 µm.*

morphologically normal dermal condensates in *Fgf20*-null skin that were readily observed underneath control placodes (Fig. 13A and data not shown). This led us to examine the expression of dermal condensate markers Sox2, *Dkk1*, and *Bmp4,* which were present in the control placode associated mesenchymal thickening but interestingly, completely absent from *Fgf20*-null skin suggesting a role for Fgf20 in the formation of the dermal condensate (Fig. 13 B-D). Further expression analysis also revealed an interesting expression phenotype of elongated stripes of *Wnt10b* in *Fgf20*-null skin implying that *Wnt10b* is negatively regulated by Fgf20 signaling directly or indirectly (Fig. 13E). Despite the possibly increased Wnt signaling, *Sostdc1* showed downregulation in expression levels in *Fgf20*-null dorsal skin (Fig. 13F). The lack of mesenchymal *Bmp4* could explain the decrease in *Sostdc1* expression.

According to the literature, there are no known mouse models with the absence of hair follicle associated dermal condensate and the molecules and cellular mechanisms guiding this mesechymal compaction are not well known. *Pdgfa-/-* mice and *lama5-/-* mice show defective dermal papilla formation but both mice reveal proper dermal condensates associated with hair placodes and buds (Guo et al. 2008; Karlsson et al. 1999). In chicks, the early dermal condensate formation has been studied more carefully. *In vitro* studies have shown that Fgf2, Fgf4, and Bmp7 are able to attract fibroblasts to form dermal aggregations (Lin et al. 2009; Michon et al. 2008; Song et al. 2004). There are no reports of Fgf2 and 4 being expressed in hair placodes. Bmp7 has been shown to be expressed in developing hairs and lack of its expression leads to the appearance of malformed follicles (Zouvelou et al. 2009). In chicks, the migration of dermal cells to form clusters may also depend on fibronectin-integrin mediated signaling, thus involving extracellular matrix-cell interactions, and stablilization of the dermal compaction may rely on integrin-Notch interaction (Michon et al. 2007).

Dermal condensate formation most likely results from physico-chemical stimuli rather than just by direct regulation by growth factors inducing the migration and clustering of fibroblasts. This hypothesis was recently further examined using mouse tooth as model (Mammoto et al. 2011). *In vitro* studies showed that mesenchymal cells were attracted by Fgf8 produced by dental epithelium but the mesenchymal compaction involves equally important repulsive signals from semaphorin 3 signaling through neuropilin 2. Further it was shown that rather than induction by Fgf8 or repulsion by semaphorin 3, the mesenchyme required mechanical compression to express odontogenic markers and to produce adequate cell density and shape combined wiht downregulation of RhoA. (Mammoto et al. 2011). It is possible that similar mechanisms are guiding dermal compaction during hair follicle (and other skin appendage) development, as well, possibly involving Fgf20 instead of Fgf8 to attract fibroblasts underneath the primary hair placodes but this requires further studies.

Our *Catnb*$^{\Delta ex3K14/+}$ mice showed increased and often continous lines of mesenchymal condensation underlying the misshapen mutant follicles, phenomenon that was observed already at E14.5, concomitant with increased Wnt and Bmp activity, which could both serve to transduce signals to form this mesenchymal phenotype. A similar effect was observed in *K5-Cre Catnb*$^{(ex3)fl/+}$ mice, as well (Suzuki et al. 2009). It would be interesting to analyse the compound mutants of *Fgf20-/-; Catnb*$^{\Delta ex3K14/+}$ to study the effect of deleted *Fgf20* on the increased dermal condensate formation upon stabilized β-catenin.

Taken together the results from the gene expression analysis of E14 *Catnb*$^{\Delta ex3K14/+}$ skin, *Fgf20-/-*, and *Sostdc1-/-* mice and previous data (Botchkarev et al. 1999; Fliniaux et al. 2008; Lefebvre et al. 2012; Kratochwil et al. 1996; Mou et al. 2006; Mustonen et al. 2004; Sick et al. 2006, Suzuki et al. 2009; van Genderen et al. 1994; Voutilainen et al. 2012; Zhang

et al. 2009) a model of molecular regulation of hair placode pattern is presented in the following and depicted in a schematic model (Figure 14). Hair placodes show high Edar and Wnt activity, which serves as placodal promoters largely by acting in a co-operative manner and having their own negative regulators. However, Eda and Wnt do interact as Wnt induces *Edar* and Eda/Edar plausibly activates *Dkk4* and maintains further Wnt activity possibly via *Wnt10a/b* upregulation. Wnts induce the expression of *Bmp2* and *Bmp4* which respond by negatively regulating Wnt activation through the inhibition of *Lef1* and *Edar* expression, as well. The placodal Bmp activity is inhibited by Ctgf and Follistatin which are both induced by Edar. Bmps and most probably mesenchymal Bmp4, induce the expression of *Sostdc1* in the epithelial surroundings of placodes where it may act by suppressing Wnt activity in interplacodal cells as lack of *Sostdc1* causes enlarged hair placodes. Due to its unique expression pattern around placodes, Sostdc1 is not behaving

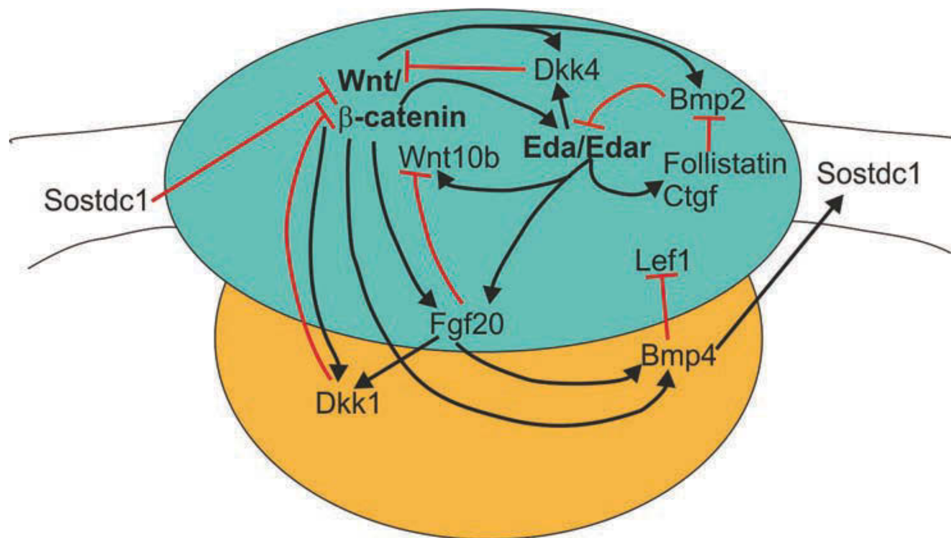

Figure 14. Molecular regulation of primary hair placode formation.

Placode formation promoting signals from placodal (blue area) Wnt/b-catenin and Eda/Edar pathways induce epithelial Fgf20 gene expression whose protein product regulates dermal marker gene expression Bmp4 and Dkk1 and dermal condensate formation (orange region). Bmps and Wnt inhibitor Dkks function to inhibit placode formation. The Eda pathway restricts Bmp activity in placodal cells by inducing Follistatin and Ctgf. Sostdc1 is induced by Bmp4 in the interplacodal region (white area) and the protein is localized to the immediate surroundings of placodes and negatively regulates Wnt activity and restricts placode size. Black and red lines indicate positive activation and negative regulation (either direct or indirect), respectively.

as a typical R-D inhibitor. The Wnt and Eda signaling both induce epithelial *Fgf20* whose protein product negatively regulates indirectly/directly *Wnt10b* expression in interplacodal region where also *Fgf20* expression itself is inhibited by an unknown mechanism. Fgf20 is

apparently involved in forming the proper physical structure of the dermal condensate and thus, may regulate indirectly or directly mesenchymal gene expression, like that of *Dkk1* and *Bmp4*. This view of the molecular regulation of placode formation suggests more complicated interactions between the regulatory players, involving more than just one activator and one inhibitor, as was earlier suggested (Sick et al. 2006).

4.3 Sustained β-catenin leads to impaired hair follicle morphogenesis, sebocyte differentiation, and hair loss but deletion of *Sostdc1* has no effect on embryonic and postnatal hair development (I, II)

The further analysis of the $Catnb^{\Delta ex3K14/+}$ mouse hair phenotype revealed severly disturbed hair follicle morphogenesis. The skin revealed densly arranged large and small hair placodes at E14.5 and enlarged buds at E15.5 invaginating from the thickened epidermis. At E17.5, the skin epidermis revealed new hair germs budding from interfollicular epidermis and also from the pre-existing hair germs. The $Catnb^{\Delta ex3K14/+}$ mutant hair buds failed to form the proper wild-type hair follicle structure characterized by a thin and long stalk with the proximal hair bulb capturing dermal papilla but rather showed further enlarged bud shape with a very short stalk and no dermal papillae were observed although dense upper dermis was still detected. In contrast, in the other two β-*catenin* mutants, *KRT14-Cre* $Ctnnb1^{(Ex3)fl/+}$ and *K5-Cre* $Catnb^{(ex3)fl/+}$ mice, follicle downgrowth and folding was arrested already around E14.5 at the placode/early bud stage after which the skin showed only thickened epidermis with impaired stratification but no signs of hair follicle-like structures (Suzuki et al. 2009; Zhang et al. 2008). This is likely due to differencies in the timing of Cre activity in these mouse models.

The embryonic skin of *KRT14-Cre* $Ctnnb1^{(Ex3)fl/+}$ and $Catnb^{\Delta ex3K14/+}$ mutants revealed dark pigmented spots but these were not described in the third model. However, all three β–*catenin* mutants showed keratinized plaques in the ectoderm which were detected more readily in the other two β-*catenin* transgenics compared to our mice (Suzuki et al. 2009; Zhang et al. 2008). Earlier studies have also shown that Wnt and Bmp signaling regulates hair keratin expression (Schlake, 2007). Suzuki and co-workers (2009) studied the Wnt induced hair follicle differentiation in *K5-Cre* $Catnb^{(ex3)fl/+}$ mice further and suggested that Wnt signals need to be transduced through Bmpr1A signaling in order to produce AE13-positive keratins, further supporting the interactive nature of Wnt and Bmp signaling in this context. Further, *KRT14-Cre* $Ctnnb1^{(Ex3)fl/+}$ mice showed increased expression levels of transcripts associated with hair shaft differentiation (Zhang et al. 2008). As a conclusion, the hair induction and hair associated keratin production requires high Wnt signaling levels but otherwise the pathway activity has to be downregulated to allow proper downgrowth of the follicle.

Previous mouse models with enhanced canonical Wnt activity, showed *de novo* formation of postnatal hair follicles with proper structure from both interfollicular epidermis and the pre-existing follicles in the normal dense fur (Gat et al. 1998; LoCelso et al. 2004; Silva-Vargas et al. 2005). This intrigued us to study advanced hair development beyond embryogenesis, as well. As the $Catnb^{\Delta ex3K14/+}$ mice die perinatally, skin transplantation was used to study the later hair morphogenetic stages. Tuft of hairs showing all four pelage hair types were detected in control skin grafts but despite continuous induction of hair development

during embryogenesis, three-and five-week old *Catnb*$^{\Delta ex3K14/+}$ skin grafts revealed almost complete hair loss. Defective hair fiber formation was observed also in the *K14Cre-Apc*$^{cko/cko}$ mice (Kuraguchi et al. 2006). The few *Catnb*$^{\Delta ex3K14/+}$ hair fibers observed showed a proper medulla structure of the analysed/observed zigzags, auchenes, and awls. Histology revealed that these hair filaments were produced by a few well-developed follicles, whereas the transplantated skin was otherwise showing impaired down-growth of other mutant hair follicles. This phenotype was markedly more dramatic compared to the *K14Cre-APC*$^{cko/cko}$ mice which were reported to have abnormal folliculogenesis and show misorientation of the follicles (Kuraguchi et al. 2006). In *Catnb*$^{\Delta ex3K14/+}$ skin, we observed a variety of other abnormalities like extra sebocytes in large clusters, radial epithelial buddings from the ORS of aberrant follicles and large epithelial cysts filled with keratin-like material. Little is known about the molecular regulation in sebaceous gland formation but signals from Wnt, Bmp, Shh, and Blimp1 regulating c-myc have been suggested to regulate the development (Schneider and Paus, 2010). In contrast to our results, earlier reports have indicated that low level of β-catenin is associated with sebocyte differentiation and high amount of canonical Wnt activity results in choice of hair lineage (Merrill et al. 2001; Lo Celso et al. 2004). Further, it was previously shown that postnatal activation of Wnt/β-catenin in skin leads to hyperkeratosis in hair follicles, (Van Mater et al. 2003) but also to hair tumor formation (Gat et al. 1998; LoCelso et al. 2004; Silva-Vargas et al. 2005) . We did not study tumor formation in our mouse model.

Observed differences in the several Wnt gain-of-function mice (Gat et al. 1998; Kuraguchi et al. 2006; Närhi et al. 2008; Suzuki et al. 2009; Zhang et al. 2008) may be explained by differences in transgene expression strength or timing. *K14ΔN87bcat* mice may have weak transgene expression due to the lack of embryonic effects on hair development. The others show early hair defects suggesting more efficient transgene activation already in embryonic skin (Kuraguchi et al. 2006**;** Närhi et al. 2008; Suzuki et al. 2009; Zhang et al. 2008). Even among these mice, variations are observed in the embryonic phenotype, our mouse model and *K14-Cre;Apc*$^{cko/cko}$ mice show less dramatic effects of hair follicle morphogenesis compared to *K5-Cre Catnb*$^{(ex3)fl/+}$ and *KRT14-Cre Ctnnb1*$^{(Ex3)fl/+}$ mice. The *K14-Cre;Apc*$^{cko/cko}$mice showed the mildest effects on hair development compared to the other three mouse models with an embryonic hair phenotype. Apc is a multifunctional protein and thus, is probably involved in other molecular regulation besides canonical Wnt signaling or its deletion may simply lead to lower level of Wnt pathway activation. All in all, these studies show the importance of Wnt signaling in the induction of hair development both in embryonic of postnatal skin but equally crucial is the control of Wnt activity during advanced developmental stages to allow normal development of hair follicle and formation of hair fiber.

As *Catnb*$^{\Delta ex3K14/+}$ skin showed dramatically disturbed hair follicle morphogenesis and hair filament production, we were curious to study the effects on embryonic and postnatal hair development upon loss of *Sostdc1*. Previous studies have shown the presence of Sostdc1 protein in the bulge and dermal papilla of postnatal hair follicles and a dynamic appearance in the mesenchymal compartment according to hair cycle progress (O'Shaughnessey et al. 2004). Further, Sostdc1 has been proposed to regulate hair cycling by controlling anagen entry by inhibiting Wnt (BeaudoinIII et al. 2005). According to our observations, however, both embryonic hair morphogenesis and the postnatal hair production were unaffected upon loss of *Sostdc1* as the knockouts showed all the four pelage hair types in expected ratios and the structure of the hair shaft medulla appeared normal, as well. Based on histological analysis of

first and second catagen stage hair follicles in anterior dorsal skin, the hair cycling remained unaffected in *Sostdc1*-null mice although a prolonged growth phase was expected. Thus, according to our results and with the knowledge that Bmp and Wnt signaling are efficiently regulated by other antagonists such as Noggin and Dkks, respectively, Sostdc1 appears to only fine-tune both embryonic and postnatal hair development.

4.4 Ablation of *Sostdc1* disturbs mammary bud development (II)

The tooth phenotype of *Sostdc1*-deficient mice has been characterized earlier and the mouse model shows extra teeth with an altered cusp pattern (Kassai et al. 2005; Munne et al. 2009; Murashima-Suginami et al. 2007). As we noticed prominent *Sostdc1* expression associated with the developing mammary glands, we were interested in examining possible *in vivo* effects of the deleted Sostdc1 on embryonic and postnatal mammary development.

Absence of Sostdc1 led to normal formation of ~E11.5 mammary placodes according to the expression pattern of the early placode marker *Lef1*. However, *Lef1*, *Wnt10b*, and *β-catenin* expression revealed that the early bud region was somewhat enlarged by E12.0-E12.5 becoming more widespread in E13.5 *Sostdc1*-null buds compared to control mice which showed smaller and more round expression pattern of the bud markers. Histological analysis and 3D-reconstructions of E13.5 mammary buds revealed altered shape with a markedly enlarged distal neck region of the mutant buds whereas the control buds showed the normal light-bulb structure. Further, the volume of *Sostdc1*-null buds was measured to be bigger than that of control buds. However, by histological analysis, these mutant-associated structural features were observed to disappear gradually prior to mammary sprout formation due to an unknown mechanism.

It has been shown that a streak of cells expressing mammary placodal markers exists briefly in development and extends from mammary placodes towards the next forming mammary rudiment (Veltmaat et al. 2003) and it has been suggested that these cells could be migrating cells on their way to the neighbouring future mammary placode (Veltmaat et al. 2004). In our studies, both E11.75-E12.25 control and *Sostdc1*-null mice showed these similar streaks between close placode/early bud neighbours as detected by *Lef1* and/or *TOP-gal* expression. However, in E11.75-E12.25 *Sostdc1*-null mice, TOPgal-positive cells were observed between buds 3 and 4, as well (see also figure 14). By E12.5-E13.0, wild-type mammary buds were detected as clear dots without any cell streaks. Interestingly, gene expression and TOP-gal reporter analysis often revealed these stripes still between neighbouring E12.5-E13.5 *Sostdc1*-null mammary buds 1 and 2, 2 and 3, and 4 and 5 as if the recruiting process of cells to the developing mammary glands was delayed. This data combined with our observations of the enlarged neck region of mutant buds and slightly increased bud volume would imply that Sostdc1 acts as a gatekeeper at the border of mammary epithelium controlling the entry of migrating cells to obtain mammary placode fate. The overall contribution of these possible extra cells appeared minor, as Sostdc1-deficient buds obtained wild-type-like shape and size by E15.5. Furthermore, as mentioned above, the observation that during wild-type bud growth, *Sostdc1* expression shifts from the epithelial borders of early buds to inside of the fully formed E15.5 buds would suggest that the ability of Sostdc1 to restrict entry of cells to mammary buds would end by that time. Moreover, the increased bud volume was apparently not a result of increased proliferation as the number of BrdU- and Ki67-positive cells within

the mammary region was similar in E12.5 and E13.5 *Sostdc1*-null and control mammary buds. On the other hand, the possible role of counterbalancing cell death to bud growth was not examined in *Sostdc1*-null mice.

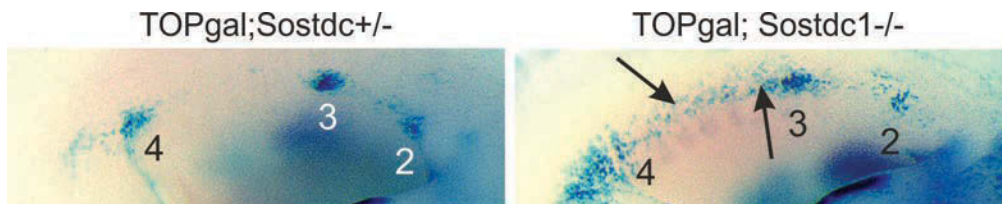

Figure 15. Ectopic Wnt activity in Sostdc1-knockout early mammary region.

X-gal staining of E11.75 TOP-gal; Sostdc1+/- and TOP-gal; Sostdc1-/- mice. Ectopic Wnt activity was detected in the Sostdc1-/- milk line between mammary placodes 3 and 4 (arrows) which was not observed in control embryos. Numbers denote the position of mammary placodes.

The cellular mechanisms behind mammary rudiment formation are still poorly known. Decades ago it was shown that mammary buds have a low mitotic index suggesting that the early development of mammary glands does not rely on proliferation (Balinsky, 1950). Later studies with rabbits implied that milk line cells migrate to the sites of forming mammary rudiments as tracing of mammary line cells "labeled" with charcoal revealed their accumulation in the placodes/early buds within 1-2 days (Propper, 1978). Recent cell-tracing experiments with BrdU-labelled cells in mice, support the idea of ectodermal influx from surface ectoderm as a first mechanism influencing the growth of mammary placodes and early buds and it is later accompanied by hypertrophy of peripheral bud cells (Lee et al. 2011). These findings support our data.

Next, we studied the molecular mechanisms behind the observed mammary bud phenotype and were particularly interested in analysing the effects of deleted *Sostdc1* on Wnt and Bmp pathway activity as Sostdc1 has been shown to modulate both signaling activities (Itasaki et al. 2003; Laurikkala et al. 2003; Yanagita et al. 2004). Earlier studies with the *Sostdc1*-null mice have suggested that Sostdc1 regulates tooth number through both pathways, although *in vivo* evidence favors only Wnt signaling (Ahn et al. 2010; Cho et al. 2011; Murashima-Suginami et al. 2008; Munne et al. 2009). In the adult kidney, Sostdc1 appears to regulate the renoprotective actions of Bmp-7 (Tanaka et al. 2006; Yanagita et al. 2007).

We observed no obvious differencies in Bmp signaling activity as assessed by pSmad 1, 5, 8 and mesenchymal *Id1* expression levels and patterns in control and *Sostdc1*-/- mammary buds. Although mammary buds showed prominent Bmp pathway activity in epithelium and mesenchyme, not much is known about the significance of these Bmp signals in early

mammary development. One *in vitro* study has proposed that Bmp signaling specifies the ventral region of mammary placodes and restricts *Lef1* expression in these mammary anlage together with *Tbx3*, but these suggestions require in vivo evidence (Cho et al. 2006).

Wnt signaling, on the other hand, has been shown to be essential for mammary placode and bud formation (Chu et al. 2004; vanGenderen et al. 1994). We observed ectopic Wnt activity in the distal epithelium of enlarged E13.0-E13.5 *Sostdc1*-null mammary buds. Interestingly, E15.5 control and mutant mice revealed similar mammary bud structure but the *Sostdc1*-null neck region displayed ectopic *TOP-gal* reporter expression partly in overlapping expression pattern to *Sostdc1*. Further, ectopic Lef1 was located in similar pattern in E12.5 and E13.5 mutant mammary buds. In consistent with our observations with enlarged early buds, previous studies have shown that forced induction of Wnt signaling in cultured embryos results in accelerated formation of enlarged mammary placodes but also to appearance of ectopic placode-like structures (Chu et al. 2004). Further, early mammary bud size was reduced upon deletion of *Lrp5* or *Lrp6* (Lindvall et al. 2009). In light of these and our findings, it would be tempting to speculate that *Sostdc1* controls correct bud size and form through negatively regulating canonical Wnt pathway. It is, however, possible that Sostdc1 regulates both Bmp and Wnt pathways during early mammary gland development but the hierarchy of the interactions between the signaling components remains unsolved at this moment.

4.5 *Sostdc1*-null mice show normal mammary ductal tree branching but exhibit extra nipple tissue with ectopic pilosebaceous units.

The suggested importance of Lrp5/6-mediated Wnt signaling in juvenile mammary ductal branching (Lindvall et al. 2009) and our observation of the strong *Sostdc1* expression in the embryonic and pubertal mammary primary sprout lead us to analyse the postnatal mammary development of *Sostdc1*-null mice. However, mutant mice showed grossly normal branching of the mammary tree during embryogenesis, postnatal puberty, and pregnancy. Whole-mount fat pad stainings for E18 embryos, three- and six-week-old mice did neither reveal extra mammary glands although *Sostdc1*-null mice showed one to three keratin 2e-positive supernumerary nipples next to the endogenous mammary glands. However, individual mammary glands showed variable penetrance of this phenotype. The extra nipples lied in close proximity to the endogenous nipples either lateral to, or in line with the mammary line and became visible after the onset of puberty when sex hormones induce the nipple growth. Histological analysis revealed that a prominent hair follicle unit with a hair filament protruding through ectoderm had replaced the lactiferous duct in the center of the ectopic nipples.

Previous studies have shown the formation of extra mammary glands when mouse skin basal cells are forced to continuously express either Eda (Mustonen et al. 2003) or Neuregulin 3 (Panchal et al. 2007). These supernumerary mammary glands with nipple and the associated mammary ductal tree arise from ectopic mammary placodes forming along the milk line (Mustonen et al. 2003, Mustonen et al. 2004) or adjacent to it, as well (Panchal et al. 2007). The induction of the ectopic *Sostdc1*-nipples, however, remains elusive. *Sostdc1*-null mice formed no extra placodes for these supernumerary nipples and the first morphological features of the embryonic mutant nipple sheaths showed no abnormalities in

size or form (data not shown). Although the supernumerary nipples were observed around six weeks of age, we would assume that the extra nipple epidermis region is determined already before puberty but due to their small size they remained undetectable before this developmental stage. Similarly, the molecular regulation affected in mutants leading to the formation of additional nipples remains unsolved in this study. Studies have shown the importance of Pthrp-Ptr1 signaling in nipple sheath determination, and the downstream regulators induced by these signals have been suggested to involve both Bmp and Wnt pathway components (Foley et al. 2001; Hens et al. 2007). *Lrp6*-knockout mice appear to have diminished embryonic nipple sheaths, suggesting a role for Wnt signaling during the embryonic determination of nipple epidermis (Lindvall et al. 2009). Nevertheless, knowing the interactive nature of the pathways, both Wnt and Bmp signals may be involved in nipple determination and it remains unsolved which pathway Sostdc1 primarily regulates in this context.

Furthermore, we often observed affected *Sostdc1*-null endogenous nipples as well, showing altered morphology with bigger size and ectopic oil red O-positive pilosebaceous units often associated with strong pigment within nipple epidermis. Despite these defects, mutant females had no obvious difficulties in nursing their pups. Previous studies have implied that Pthrp positively regulates Bmp signaling which stimulates *Msx2* expression in primary mammary mesenchyme to prevent hair follicle formation in the nipple skin. Furthermore, ablation of Msx2 rescued the formation of hair follicles in the K14-*Pthrp* ventral skin (Hens et al. 2007). Similarly, another study suggested that the Bmp pathway prevents hair follicle fate among nipple epidermal cells, as overexpression of Noggin in skin basal cells caused ectopic pilosebaceous follicle formation in nipple epidermis (Mayer et al. 2008). These transgenic mice, however, did not reveal ectopic nipple tissue or dysformed morphology and otherwise showed a similar phenotype to our knockout mice. If Bmp activity protects nipple skin from hair follicle formation and Sostdc1 negatively regulates Bmps in this context, then loss of *Sostdc1* should not have resulted in ectopic pilosebaceous unit formation. Perhaps, Sostdc1 prevents pilosebaceous follicle appearance in the nipple region by inhibiting Wnt activity, which is known to positively regulate hair follicle formation (Gat et al. 1998). Further, histological analysis of few E14 mammary buds revealed the occasional appearance of hair germ-like structures within the inhibitory region around mammary buds and a similar intrusion of hair placodes, and this was more dramatically observed in the *Catnb*$^{\Delta ex3K14/+}$ mice.

Humans show familial or sporadic supernumerary nipples, which is a rather common condition and usually considered as a cosmetic problem (Grossl, 2000). Ectopic nipples have been classified to eight types according to Kajava (1915) detected along the presumptive milk line between the axilla-pubic and arising with or without associated mammary gland, conditions known as polymastia or polythelia, respectively. They may serve as a sign of defects in other organs, like kidney and urinary tract abnormalities (Grossl, 2000; Brown and Schwartz, 2004). The Simpson-Golabi-Behmel syndrome is caused by mutated *GPC3* and affected patients have supernumerary nipples in addition to congenital heart defects and polydactyly (Veugelers et al. 2000). It is tempting to speculate that mutated *Sostdc1* could be a cause for human polythelia in some conditions.

5 CONCLUDING REMARKS

Earlier studies have suggested essential roles of Wnt signaling in skin appendage development. Thus, we were curious to further examine the significance of this pathway on the formation of hair and mammary glands and to study the downstream molecular targets of the pathway especially during early hair formation to understand better the regulation of placodal patterning. Three mouse models were used in this study, in which Wnt signaling was modulated by stabilising β-catenin in skin epithelium, deleting the Wnt and BMP pathway modulator, *Sostdc1*, or ablating the Wnt target gene, *Fgf20*. The early developmental steps were analysed and postnatal morphogenesis was studied.

We observed that *Sostdc1* has a dynamic expression pattern in hair and mammary gland development, and similar to mouse tooth showed a rather unique localization in the epithelial surroundings of the organ primordia different from other skin appendage placodal or bud marker genes. Despite the dramatic effects of Sostdc1 on cusp pattern and the number of teeth, the loss of this factor caused milder phenotype change in other skin appendages. Hair development was grossly normal, although extra sensory hairs in the snout skin were observed. Interestingly, early mammary gland development was affected upon loss of Sostdc1 showing increased mammary bud size with altered shape. Postnatally, extra nipple tissue with ectopic pilosebaceous follicle-like structures were detected, as well. However, the ectopic nipples were not associated with the mammary tree, however. The question whether Sostdc1 regulates the vibrissa and mammary bud/nipple formation primarily through the Bmp or Wnt pathway, remains unsolved in the light of these studies. However, based on prior knowledge about the roles of these two signal pathways in hair and mammary gland development and our observation of ectopic Wnt activity/Lef1 expression and unchanged Bmp activity in *Sostdc1-/-* mammary buds it is tempting to suggest a Wnt-Sostdc1 interplay. Bmps may still be involved in inducing *Sostdc1*, as suggested during early pelage hair development, as Wnts are thought to be unable to do this. Knowing the dynamic expression pattern of this modulator that we learned during this study, the soluble nature of Sostdc1, and the interactions between Bmp and Wnt, the hierarchy of the three components may vary according to different developmental stages and thus, is difficult to study at least by conventional tissue culture methods. Some idea to the molecular mechanisms could be provided by the analysis of *Sostdc1-/-;Lrp5/6-/-* and *Sostdc1-/-; Bmpr1A* $^{fl/+}$ compound mouse mutants to determine whether decrease of Wnt or Bmp signaling is able to rescue the observed vibrissa and/or mammary phenotype.

Even though prominent *Sostdc1* expression was observed in the embryonic and postnatal primary duct, the absence of Sostdc1 resulted in normal mammary gland branching, suggesting compensatory actions by other regulators. In a recent study, SOSTDC1 mRNA and protein expression was detected in normal human breast tissue by microarray and immunostained tissue microarray, respectively. Interestingly, mRNA and protein expression levels were downregulated in breast cancer patients. Moreover, SOSTDC1 protein levels appeared to diminish upon an increase in breast tumor size. Further, in the study using

well-characterized breast cancer cell lines it was shown that SOSTDC1 suppressed BMP7-mediated activation of Smad phosphorylation but did not affect the actions by BMP2 and Wnt3a. (Clausen et al. 2011). These findings suggest a role for Sostdc1 as a tumor suppressor in breast cancer but the molecular mechanism behind this pathological condition needs to be analysed further.

A dramatic hair phenotype was observed in mouse mutants with stablilized β-catenin leading to ectopic and excessive hair placode formation. Further, comparing the various studies with Wnt gain-of-function mice, our *Catnb*$^{\Delta ex3K14/+}$ mouse model was the first one to present precocious hair development and suggested that Wnt is both necessary and sufficient to induce hair development even in the absence of the early placode promoter Eda-Edar signaling, placing Wnt signaling above all other placodal regulators. Further, we suggest that Wnts induce Bmp signaling and that the interplay of the two pathways produces correct hair placode patterning in addition to other suggested activator-inhibitor pairs implying that hair induction is guided by complex regulatory systems. Although, the Wnt activity was sufficient for hair induction even in hairless regions, the sustained canonical Wnt signaling disturbed subsequent hair morphogenesis and hair production.

For future hair development studies, we introduce an interesting new mouse model, the *Fgf20-/-* mouse, showing the absence of proper dermal condensate morphology and associated expression of several markers; however, this needs to be further analysed. It may serve as the first mouse model to show the loss of complete dermal condensate and thus, provides an interesting tool to study the formation of this mesenchymal compartment but raises questions about the early patterning of hair placodes. The *Fgf20-/-* placodes were able to form correct morphology and normal patterning even in the absence of several mesenchymal signals and visible dermal condensate, thus indicating that placodes are first to appear before the accompanying dermal compartment.

Based on over 40-year old tissue recombination studies (Sengel, 1976) it has been believed that only dermis is possesses the intrinsic ability to initiate cutaneous appendage development as dermis from the non-haired region was not able to induce hair formation in normally competent epidermis. Our observations suggest, however, that *Fgf20-/-* epithelium is able to pattern itself without mesenchymal patterning and thus, also would imply that the first developmental signal could arise in epithelium, similar to tooth (Mina and Kollar, 1987). Perphaps the various tissue recombination experiments do not fully cover all the possible outcomes and thus, leaves a gap to deduction. Especially it would be intriguing to know the result of combining epidermis from non-haired region with non-patterned dermis (E12.5-E13) from a haired region, since in previous studies already patterned dermis with hair inductive signals was used (Dhouailly, 1973; Kollar, 1966; Kollar 1970). Assuming that the epithelium provides the first signal, no hair placodes should be induced in glabrous epithelium in this suggested experimental set-up as neither mesenchyme nor epithelium is able to give signals. It may also be that the glabrous epithelium possesses all the necessary hair inductive signals but the dermis from similar skin regions provides a cocktail of inhibitors suppressing hair formation in the epidermis but this has not been studied. In this case the presented recombination set-up would lead to hair formation when competent epidermis would be combined with competent dermis.

In the case that the epithelium is capable of producing the first crucial signal(s) leading to hair formation and as stabilized β-catenin was able to induce precocious and ectopic hair induction in haired and non-haired regions it could be that the Wnt pathway serves

as the first initiative signal leading to epithelial *Fgf20* induction and formation of dermal condensation. Further, it would be interesting to know whether Fgf20 is mediating direct effects when orchestrating dermal condensate formation or are the signals sent secondarily through another pathway. It would also be interesting to know what cellular mechanims Fgf20 induces, as Fgfs are known to promote both proliferation and migration. *p21,* a marker for cell differentiation, is known to be expressed in the forming dermal condensates we observed (data not shown) and the proliferation status of dermal condensate has been shown to be very low (Sengel, 1976). These findings would suggest that the migration of dermal cells serves as a mechanism in the early stage of dermal compaction. It requires further studies to investigate whether Fgf20 functions similar to Fgf8 in tooth mesenchyme formation (Mammoto et al. 2011), i.e. in attracting fibroblasts to migrate underneath placodes.

Moreover, the *Fgf20*- knockout mouse model is useful in examining the hierarchy of signals regulating primary hair placode patterning due to the absence of functional dermal condensate. More studies are required to solve whether Fgf20 regulates subsequent hair waves similarly or if it is replaced by other Fgfs or growth factors from other families. Furthermore, the morphogenesis of hair follicles arising from the three different hair waves would be worth analysing as the absence of dermal condensates may affect later development and perhaps hair filament production. In addition, hair cycling apparently involves signals from the Fgf pathway although the precise mechanism is not known, but Fgf20 may control the cyclic hair follicle rest and regeneration stages. Also the analysis of hair types and density would be of interest to study.

Knowing the signaling pathways and the hierarchy and network of the signals and transcription factors guiding the induction and morphogenesis of embryonic hair follicles may help us to understand how new hair follicles could be produced and used as a therapy to treat hair loss in humans. This thesis work emphasizes the important function of canonical Wnt signaling through Fgf20 in hair induction but shows also the importance of tight control of Wnt pathway activity to allow proper hair follicle and hair filament formation. Further, we gained some new insights to the hierarchy of early signals in embryonic hair development. In a broader context, discovering the complex gene regulatory networks underlying skin appendage formation may teach us about similar molecular mechanisms governing other organ development, as well.

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