The role of Lhx2 in hair follicle morphogenesis and regeneration

Gunilla Törnvist
Table of Contents

Abstract 5
Paper in this licentiate thesis 7
Abbreviations 8
Introduction 9
  The hair follicle 9
  Hair follicle morphogenesis 10
    Molecular regulation of the HF morphogenesis 12
  The hair cycle 13
    Catagen 14
    Telogen 15
    Anagen 15
  LIM-homeodomain transcription factors 18
    Lhx2 19
Aim 21
Results and discussion 22
  Expression of Lhx2 during HF morphogenesis and postnatal cycling 22
  Transgenic over-expression of Lhx2 in HFs results in anagen induction 23
  Mice with inactivated Lhx2 are unable to regenerate a normal hair shaft 28
  Significantly reduced levels of Lhx2 during morphogenesis 30
  Lhx2 and the HF stem cells 32
    How does Lhx2 function in the HFs? 35
    Final conclusions 36
Enkel svensk sammanfattning 38
Acknowledgements 40
References 41
Paper I
Abstract

Hair is important for thermoregulation, physical protection, sensory activity, seasonal camouflage and social interactions. Hair is produced in hair follicles (HFs), complex mini-organs in the skin devoted to this task. HFs are formed during embryonic development (morphogenesis) and new hair is continuously generated throughout life since the postnatal HF goes through cycles of regression (catagen), quiescence (telogen) and growth (anagen). The transcriptional regulation of this process is not well understood. The LIM-homeodomain transcription factor Lhx2 has previously been shown to be critically involved in epithelial-mesenchymal interactions during development of various organs and a potent regulator of stem cell function. We therefore elucidated the expression pattern and function of \( Lhx2 \) during hair formation.

\( Lhx2 \) is expressed during both morphogenesis and anagen in cells scattered in the outer root sheath and in a subpopulation of the matrix cells in the proximal part of the hair bulb. Matrix cells are proliferating progenitor cells that differentiate into the components of the HF including the hair shaft. Expression is turned off during telogen, however \( Lhx2 \) expression reappears in the secondary hair germ immediately prior to initiation of the anagen stage. In contrast to previously published results \( Lhx2 \) appears to be expressed by progenitor cells distinct from those in the stem cell niche in the bulge region. The developmental-, stage- and cell-specific expression pattern of \( Lhx2 \) suggests that Lhx2 is involved in the generation and regeneration of hair.

To test our hypothesis we used different genetically modified mouse strains. First we studied the effect of over-expression of \( Lhx2 \) in the HFs using a mouse model where transgenic Lhx2 expression could be induced in dorsal skin. Using this model we could show that \( Lhx2 \) expression is sufficient to induce anagen. To analyze the consequence of lack-of-function of Lhx2 we developed a mouse model where it is possible to conditionally inactivate \( Lhx2 \) and a mouse strain harbouring a hypomorphic allele of Lhx2. Mice where \( Lhx2 \) was conditionally inactivated in postnatal HFs were unable to regrow hair on a shaved area whereas all controls did regrow their hair. The mutant HFs initiated anagen but were unable to produce normal hair shafts. Thus Lhx2 is required for postnatal...
hair formation. We used the mouse strain carrying a hypomorphic allele of \textit{Lhx2} to study the role of Lhx2 during HF morphogenesis. Embryos homozygous for the hypomorphic allele form significantly less HFs compared to control embryos, and the HFs that do form in the mutant embryos appear to be developmentally arrested. These results suggest that Lhx2 is also important during HF morphogenesis. Thus, Lhx2 is an essential positive regulator of hair generation and regeneration.
Paper in this licentiate thesis

This thesis is based on the following paper that will be referred to in the text by its roman numeral I.


*The two first authors contributed equally to this work.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>APM</td>
<td>arrector pili muscle</td>
</tr>
<tr>
<td>CTS</td>
<td>connective tissue sheath</td>
</tr>
<tr>
<td>DP</td>
<td>dermal papilla</td>
</tr>
<tr>
<td>E</td>
<td>embryonic day</td>
</tr>
<tr>
<td>HD</td>
<td>homeodomain</td>
</tr>
<tr>
<td>HF</td>
<td>hair follicle</td>
</tr>
<tr>
<td>HG</td>
<td>hair germ</td>
</tr>
<tr>
<td>2°HG</td>
<td>secondary hair germ</td>
</tr>
<tr>
<td>IRS</td>
<td>inner root sheath</td>
</tr>
<tr>
<td>K</td>
<td>keratin</td>
</tr>
<tr>
<td>ORS</td>
<td>outer root sheath</td>
</tr>
<tr>
<td>P</td>
<td>postnatal day</td>
</tr>
<tr>
<td>SG</td>
<td>sebaceous gland</td>
</tr>
<tr>
<td>Tx</td>
<td>tamoxifen</td>
</tr>
</tbody>
</table>
Introduction

The hair follicle

Hair is one of the distinguishing features of mammals and its functions include thermoregulation, physical protection, sensory activity, seasonal camouflage and social interactions. It is produced by hair follicles (HFs) which are complex mini-organs of the skin. The human body displays about 5 million HFs, of which 80,000 to 150,000 are located on the scalp. Few, if any, additional HFs are formed after birth, however, individual HFs can change drastically over time. For example, thicker and darker hairs replace the fine lightly pigmented vellus hairs in the beard at puberty for boys and thick scalp hairs convert into vellus hairs later in life. The HF is also unique as it throughout life undergoes cycles of rapid growth (called anagen), regression (catagen) and rest (telogen). The length of the growth phase is determining the length of the hair, and anagen for scalp hair lasts for between 2 and 6 years while it is considerably shorter for eyebrows. Hair disorders, both in form of hair loss and unwanted hair growth, are not life threatening although of major inconvenience for the affected individual. That is one reason for the importance in studying the HF. Furthermore, the HF is an outstanding model system for several questions in developmental biology as it is an autonomous organ that is well-defined, easily accessible and is repeatedly regenerating (Cotsarelis 2001; Krause 2006).

Figure 1: The hair follicle. A) A HF in its growing phase. B) A close-up of the hair bulb showing the eight endothelial HF layers and the surrounding mesenchymal CTS. APM, arrector pili muscle; CTS, connective tissue sheath; DP, dermal papilla; IRS, inner root sheath; ORS, outer root sheath; SG, sebaceous gland. A is adopted from Müller-Röver (2001) and B from Schneider (2009).
The structure and function of the HF is conserved between species, and in this licentiate thesis the mouse is used as a model organism. The HF is composed of numerous cell types. The epidermal portion of the HF consists of the outer root sheath (ORS), the inner root sheath (IRS) and the bulb. The hair bulb is located at the proximal portion of the HF and contains the matrix cells (Figure 1A). The growing hair shaft is the result of differentiating matrix cells and the final hair shaft is composed of terminally differentiated, dead cells. In pigmented animals melanin are synthesized by melanocytes located close to the forming hair shaft. Separating the ORS and the IRS is the companion layer, and the IRS is divided into Henle’s layer, Huxley’s layer and the IRS cuticle. Innermost of the eight cell layers composing the epidermal HF cylinder are the medulla, cortex and hair cuticle of the hair shaft (Figure 1B). All layers can be defined from their expression pattern of specific proteins, especially different keratins (Fuchs 2007; Schneider 2009). Enveloped by the epidermal cells of the bulb is the dermal papilla (DP), one out of two mesenchymal components of the HF. The second mesenchymal component is the connective tissue sheath (CTS) surrounding the HF, and the mesenchyme is separated from the skin epithelium by a basement membrane of extracellular matrix. In the distal portion of the HF is the sebaceous gland (SG) located and just below, at the attachment site for the arrector pili muscle (APM), is a convexity of the ORS called bulge which contains HF stem cells (Figure 1A; Müller-Röver 2001; Schneider 2009). The bulge stem cells are slow cycling and hence defined by their label-retaining properties (Cotsarelis 1990) and express keratin 15 (K15) and CD34 (Liu 2003; Trempus 2003). The HF stem cells are capable of reconstructing the HFs and of wound repair in the interfollicular epidermis (Blanpain 2004; Ito 2005).

**Hair follicle morphogenesis**

The HFs in mice initiate morphogenesis at different time points during embryonic development depending on hair type. The pelage (fur) hair is divided into primary and secondary HFs. The tylotrich (also known as primary or guard) HFs produce extra hard and long hairs that have a sensory function and comprise 1-3 % of the coat. Their development starts around embryonic day (E) 14. There are three types of non-tylotrich HFs, producing awl, auchene and zigzag hairs. Zigzag hairs are the most common type,
comprising 65-70 % of the pelage hairs, and are characterized by three to four hair shaft bends. Auchene hair shafts have one bend and comprise 0.1 % of the HFs and awl hairs are straight and comprise 30 % of the hair coat. The morphogenesis of awl and auchene HFs start at E16 to E17, and of zigzag hairs at E18 to P1. An additional hair type is the whiskers which are very large and have a tactile function, and whisker HF morphogenesis starts around E12.5 (Duverger 2009).

HF morphogenesis has been divided into distinct phases denoted stage 0-8 (Figure 2; Paus 1999). The first stage, stage 0, corresponds to the undifferentiated and single-layered epidermis with no morphological signs of HF induction. At stage 1 a thickening of the epidermis called placode appears. The adjacent dermal fibroblasts are changing their orientation and are gathering, and will form a dermal condensate during the next stage when the hair placode elongates into the primary hair germ (HG). At stage 3 the epidermal part of the HF has formed a hair peg and the epidermal cells are arranged as a column. The dermal condensate eventually develops into the DP, and when the hair peg continues to grow into the
dermis (stage 4) it displays a proximal bulb-like thickening that begins to envelope the DP. At stage 4 the IRS also starts to develop and appears as a cone over the DP. At stage 5 the HF has developed into a bulbous hair peg and the first melanin granules are visible above the DP (if the mouse is pigmented), and the bulge becomes discernable. At stage 6, the HF has grown down into the subcutis layer of the skin, the hair shaft has formed and the DP is completely enclosed by epidermal cells. During stage 7, the tip of the hair shaft enters the hair canal. Finally, at stage 8 the HF reaches its maximum length and the bulb is adjacent to the subcutaneous muscle layer, and the hair emerges through the epidermis (McElwee 2000; Paus 1999).

**Molecular regulation of the HF morphogenesis**

Follicular morphogenesis is dependent on epithelial-mesenchymal interactions. The first signal to start HF development is thought to come from the dermis but what this first dermal signal consists of is still to be defined (Hardy 1992). However, numerous signaling molecules have been identified as essential for initiation of HF morphogenesis. Canonical Wnt/β-catenin signaling in the surface ectoderm is central for epidermal cells to adopt HF fate. When the Wnt inhibitor Dickkopf1 (Dkk1) is ectopically expressed in the epithelium or epithelial β-catenin expression is conditionally targeted, it results in a complete block in HF formation (Andl 2002; Huelsken 2001). The DNA-binding protein Lef1 is normally forming a complex with stabilized β-catenin to activate transcription of target genes, and mice with an inactivated Lef1 gene develop almost no HFs in the back skin (van Genderen 1994). Furthermore, over-expression of a stabilized form of β-catenin causes ectopic HFs within the interfollicular epidermis (Gat 1998). In contrast to Wnt signaling, which is activated during placode formation, bone morphogenic protein (BMP) signaling must be inhibited for placode morphogenesis to progress. In absence of the BMP-inhibitory factor noggin HF density is reduced (Botchkarev 1999a). Furthermore, signaling pathways have to elicit changes in cell junction proteins in the original single-layered epidermis for the placodes to be able to form. The critical cell-cell adhesion protein E-cadherin is downregulated during this process (Hirai 1989; Müller-Röver 1999). The E-cadherin promoter harbors multiple Lef1 binding sites and Lef1, as well as Shh, is induced by noggin, indicating a connection of the signaling pathways during HF formation (Botchkarev 2001; Jamora 2003).
Once the placodes have formed, sonic hedgehog (Shh) is required for further development. When Shh binds to its receptor Patched1 (Ptc1), the inhibitory effect of Ptc1 on Smoothed (Smo) is released leading to transcription of specific target genes including Gli1 and Ptc1 (Millar 2002). Without Shh expression, HF morphogenesis arrests at the placode stage and the underlying dermal cells fail to condensate suggesting a role in the epithelial-mesenchymal crosstalk and for cell proliferation (Chiang 1999; St-Jacques 1998). Shh has also shown to be involved in the polarization of the developing HF (Hammerschmidt 2007). Moreover, some signaling pathways appear to affect the morphogenesis of specific hair types differently. The tylotrich HFs are dependent on the growth factor ectodysplasin (Eda) and its receptor Edar for their initiation. Deletion of either Eda or Edar leads to failure in induction of guard HFs whereas the other hair types develop normally (Headon 1999). Eda and Edar act downstream of Wnt signaling (Laurikkala 2001), and NK-κB is required downstream of Eda and Edar to activate Shh expression (Schmidt-Ullrich 2001; Schmidt-Ullrich 2006).

The hair cycle

After morphogenesis the HF is repeatedly regenerated as it cycles through stages of regression (catagen), quiescence (telogen) and growth (anagen; Figure 3A). The reasons for this complex regulation is not clear but could be that it provides a mechanism for adapting to seasonal variations (e.g. changing from summer to winter coat) and making it possible to control the length of body hair uniquely from site to site (Paus 2004; Stenn 2001). In mice, the first cycles of back skin HFs are synchronized and anagen initiates in waves that sweep in a posterior to anterior direction. With age the waves become less frequent as telogen duration extends and also they become less synchronized and eventually anagen initiates in local patches. The first catagen is initiated when the mice are just over two weeks old, lasting for two to three days and followed by telogen. Four weeks after birth the first anagen stage is initiated (Figure 3B). However, the time scale can differ slightly depending of genetic background and sex as well as environmental and nutritional factors (Müller-Röver 2001).
Figure 3: The hair cycle. A) The HF is cycling through stages of growth (anagen), regression (catagen) and rest (telogen). B) Time-scale for the hair cycle during the first 14 weeks after birth, based on female C57BL/6 mice. Adopted from Müller-Röver (2001).

Catagen

Morphogenesis of HFs in back skin continues after birth for two weeks until the hair shafts reach their maximum length. Subsequently the HFs enter catagen and start to degrade the lower two-thirds of the HF in an apoptosis driven process. The first signs of catagen induction are disrupted pigmentation of the hair shaft and narrowing of the bulb (Figure 4A). The HF becomes shorter and an epithelial strand is formed between the DP and the germ capsule, surrounded by a thick glassy membrane (Figure 4B). The hair shaft is transformed into a club hair and moves distally until the proximal part reaches the bulge. The germ capsule and the DP are crossing the border to the dermis followed by the trailing CTS in the subcutis (Figure 4C; Müller-Röver 2001; Paus 2004). Catagen must be regulated very strictly to secure that apoptosis not depletes the epithelial cell pools from which the new anagen hair bulb is constructed since that would endanger the HF’s capacity to regenerate. Important for this is the transcription factor Hairless (Hr). Hr functions as a negative transcriptional repressor and guarantees that apoptosis occurs in the correct tissue components, and in the absence of
functional Hr the HF is destroyed during the first catagen phase leading to complete hair loss (Montagna 1952; Panteleyev 1998; Panteleyev 1999). Fibroblast growth factor 5 (FGF5) is important for the transition from anagen to catagen. Constitutive inactivation of the FGF5 gene in mice results in prolonged anagen phase leading to an angora phenotype (Hébert 1994). Expression of the neurotrophin proteins NT-3, NT-4 and brain-derived neurotrophic factor (BDNF) is upregulated in the HF epithelium prior to catagen, and mice transgenic for either one of the three neurotrophin factors show premature catagen induction (Botchkarev 1998; Botchkarev 1999b). Furthermore, transforming growth factor β1 (TGF-β1) is able to induce premature anagen termination when administrated to mouse skin and deletion of the gene leads to a delay in catagen onset (Foitzik 2000). K17 works together with NF-κB, a downstream effector of tumor necrosis factor α (TNF-α) signaling, to regulate the rate of apoptosis and thus controlling catagen entry (Tong 2006).

Telogen

Degradation is followed by a period of relative quiescence termed telogen (Figure 4D). In telogen the HF reaches its minimal size. The whole HF resides in the dermis and the remaining hair shaft is a club hair, unpigmented in the proximal portion. Only the permanent, non-cycling upper HF is represented, with SG, APM and bulge, while there are no IRS or bulb structures. The DP is separated from the bulge by the secondary hair germ (2°HG; Müller-Röver 2001). Telogen is a stage of relative quiescence, however not of complete rest (Paus 2004). Plikus et al. (2008) found that BMP2 and BMP4 are expressed in dermal adipocytes during the beginning of telogen but then are downregulated during late telogen. This fluctuating expression is dividing telogen into two phases: refractory and competent telogen. HFs in competent telogen allow the anagen-reentry wave to propagate, and HFs in refractory telogen arrests the wave. Consequently, in mice which over-express the BMP inhibitor noggin after a K14 promoter the telogen phase is drastically shortened (Plikus 2008).

Anagen

Anagen is divided into eight substages (Figure 4E-L). The first morphological sign of anagen induction, at substage I, is a thickening of the 2°HG due to proliferation of the 2°HG cells. The 2°HG continues to
Figure 4: Detailed presentation of three catagen substages (A-C), telogen (D) and the eight anagen substages (E-L). APM, arrector pili muscle; CTS, connective tissue sheath; DP, dermal papilla; IRS, inner root sheath; ORS, outer root sheath; SG, sebaceous gland.

elongate and thicken and the epidermal cells begin to enclose the DP during substage II, and during substage IIIa the hair bulb is formed by matrix cells, the IRS appears as a cone of keratinized cells above the DP and the first melanin granules appear. The bulb is starting to cross the border between the dermis and the subcutis. At substage IIIb a pigmented hair shaft starts to grow. The bulb reaches its maximum size during substage IIIc and the ORS becomes clearly distinguishable from the IRS. During substage IV the tip of
the IRS and the new hair shaft reaches the hair canal and this may result in shedding of the club hair but the club hair can also remain during several cycles. At substage V, the IRS stops to grow at the level of the SG and the hair shaft continues to the hair canal. During the final substage VI the HF is fully developed, the DP entirely enveloped in the bulb residing adjacent to the subcutaneous muscle layer, and the hair shaft emerges through the epidermis (Müller-Röver 2001).

Given the morphological similarities between anagen and HF morphogenesis it is not surprising that there is a considerable overlap in the pathways promoting the both processes. Signaling through Wnt/β-catenin/Lef1 and Shh as well as inhibition of BMP are important for HF morphogenesis as well as anagen. Wnt10, Wnt10b and the Wnt receptor Frizzled are prominently upregulated in the HF epithelium at anagen onset, and transient activation of β-catenin in telogen skin induces anagen (Reddy 2001; Reddy 2004; van Mater 2003). Modulation of BMP4/BMPR-IA signaling is required for anagen induction, and the BMP inhibitor noggin is consequently upregulated in the HF epithelium and mesenchyme in the end of telogen (Botchkarev 2001). Enhanced expression of the Shh gene in postnatal skin accelerates initiation of anagen (Sato 1999). However, neutralization of Shh using an anti-Shh antibody results in disrupted hair regeneration but anagen is still initiated, which indicates that Shh, as during HF morphogenesis, is required for the proliferation of epithelial cells after onset of anagen (Wang 2000). Moreover, several other proteins have been identified as important for anagen induction. For example is deletion of STAT3 transcription factor associated with a long-term delay of anagen initiation (Sano 1999), and injection of PDGF isoforms into skin immediately induces anagen (Tomita 2006). Expression of hepatocyte growth factor (HGF) is observed in the DP fibroblasts and its receptor Met is expressed by neighboring hair bulb matrix cells during early and mid anagen indicating a role in epithelial-mesenchymal cross-talk, and local injection of HGF into skin prolongs anagen (Jindo 1998; Lindner 2000).

Once the anagen phase is initiated and the HF has started to grow, matrix cells proliferate and differentiate into the cell lineages of the IRS and the hair shaft. The matrix is suggested to contain progenitor cells committed to each inner sublayer of the HF (Legué 2005). The Notch pathway is known to regulate cell fate specification and patterning during development of diverse tissues, and is important for patterning of the matrix
cells (Lai 2004; Lin 2000). Gata3 is a Notch target expressed in the IRS, and inactivation of Gata3 leads to failure of the differentiation of IRS precursor cells (Amsen 2007; Kaufman 2003). The transcription factor Dlx3 have been found to position downstream of Wnt signaling in the regulatory cascade involved in HF differentiation and upstream of Hoxc13 and Gata3 (Hwang 2008). When Dlx3 expression is removed from the epidermis the result is complete alopecia due to failure of the hair shaft and the IRS to form (Hwang 2008). The transcription factors Msx2 and Foxn1 are found to function upstream of Notch1 and as they also contribute to the expression of keratins in the cuticle and cortex of the hair shaft, they are important for IRS and hair shaft differentiation. Msx2−/− mice exhibit cycles of hair loss and regrowth and structurally abnormal hair shafts (Ma 2003), and mice with a mutated Foxn1 gene, called nude mice, have structural defects in hair shaft and IRS leading to fragile hairs that rarely penetrates the skin surface (Nehls 1994). Msx2/Foxn1 double mutants lack all hairs (Cai 2009). BMP signaling is required for the differentiation of the HF and act upstream of Msx2 and Foxn1 (Cai 2009). A decrease in BMP signaling by ectopic expression of noggin, or inactivation of the BMP receptor BMPR1a, results in impaired differentiation of the IRS and hair shaft (Kobielak 2003; Kulessa 2000). Moreover, Wnt signaling appears to play a role in HF differentiation since Lef1 is expressed in the hair shaft precursor cells and over-expression of Wnt3 in transgenic mouse skin causes a short-hair phenotype due to altered differentiation of hair shaft precursor cells (DasGupta 1999; Millar 1999).

A wide range of factors, of which a selection has been discussed above, have been identified to regulate the hair cycle mainly by looking at expression patterns in different follicular compartments at distinct hair cycle stages and by studying mutant mice with defects in HF cycling. However, more signaling pathways and transcription factors have to be studied to get closer to the whole picture.

**LIM-homeodomain transcription factors**

One group of transcription factors that plays a critical role during the development of several different organs is the LIM-homeodomain (LIM-HD) transcription factors. LIM-HD proteins consist of two LIM-domains and a HD. The LIM domain is named by the initials of the three HD proteins
in which it was first discovered, Lin11, Isl-1 and Mec-3 (Freyd 1990; Karlsson 1990; Way 1988), and is a cysteine-histidine rich domain containing two tandemly repeated zinc fingers. The combination of protein binding LIM domains and a DNA binding HD gives the transcription factor a unique potential to combinatorially interact with other transcriptional regulators (Bach 2000; Curtiss 1998; Hobert 2000).

LIM-HD transcription factors have been well preserved throughout evolution and exist in diverse species from invertebrates like *C. elegans* and *Drosophila* to mammals. They have been shown to play important roles in cell fate decisions and organ development (Curtiss 1998; Hobert 2000). Several have shown to be involved in early patterning of the embryo, including Lim-1 that is necessary for initial patterning of *Xenopus* and has a head-organizer function in mice (Shawlot 1995; Tiara 1992) and apterous, which is essential for the dorsoventral patterning of the *Drosophila* wing (Cohen 1992; Diaz-Benjumea 1993). Furthermore, LIM-HD proteins are often involved in the differentiation of different tissues and cell types. For example, Mec-3 is required for differentiation of the touch receptor neurons of *C. elegans* (Way 1988) and apterous is involved in the specification of a subset of larval muscle precursors and in the specification or differentiation of some neurons in the CNS (Bourgouin 1992; Lundgren 1995). As shown for apterous, a LIM-HD transcription factor can function in development of different organs of the organism (Bach 2000; Hobert 2000).

The LIM-HD proteins are divided into six groups according to their sequence homologies; the LIN-11, Apterous, Lim-3, Islet, Lhx6/Lhx8 and Lmx groups (Hobert 2000). Members of the Apterous group are the *Drosophila* apterous gene, the *C. elegans* ttx-3 gene and the two vertebrate genes Lhx2 and Lhx9. The two latter are closely structurally related and their expression patterns are partially overlapping (Bertuzzi 1999; Porter 1997; Rétaux 1999). For this licentiate thesis, Lhx2 is of particular interest.

**Lhx2**

LIM-homeobox gene 2 (*Lhx2*; previously called *LH-2* or *LH2A*) was first identified as a pre-B-cell-specific gene (Xu 1993). It is expressed during development and diverse functions are reported. Embryos lacking *Lhx2* expression show a diminished forebrain due to hypoplasia of the cerebral
cortex and agenesis of the hippocampal anlagen (Porter 1997). Lhx2 appears to be crucial for patterning of the telencephalon (Bulchand 2001; Monuki 2001). Furthermore, Lhx2 is essential for eye formation. In the null embryos eye development arrests prior to the formation of an optic cup (Porter 1997). Lhx2 is also expressed in the progress zone of the growing limbs, however the limbs are developing normally in Lhx2\(^{-/-}\) mice since the LIM-homeobox genes Lhx9 and Lmx1b compensate for Lhx2 loss (Tzchori 2009). Lhx2 is also expressed in the olfactory epithelium and required for differentiation of progenitors into mature olfactory sensory neurons (Kolterud 2004a).

The liver in the Lhx2 mutant is substantially reduced in size (Porter 1997), however a liver is still formed showing that Lhx2 is not involved in initiation of liver development. Lhx2 is expressed in the septum transversum mesenchyme surrounding the forming liver bud. After liver formation through intermingling of hepatic cords into the septum transversum mesenchyme, Lhx2 expression is maintained in a subpopulation of mesenchymal cells in the liver, the hepatic stellate cells (Kolterud 2004b). Stellate cells are involved in liver fibrosis, and the livers in the Lhx2\(^{-/-}\) embryos are fibrotic and severely disorganized, suggesting that Lhx2 inhibit fibrosis development and this is important for proper formation of the liver (Wandzioch 2004). From mid gestation to around birth, the fetal liver is the main hematopoietic site (Cumano 2007), and Lhx2\(^{-/-}\) mice consequently have an impaired hematopoietic environment causing a severe anemia and the embryos die \textit{in utero} at E15 to E17 (Porter 1997). Moreover, it has also been shown that Lhx2 can regulate hematopoietic stem cell (HSC) function since expression of Lhx2 in fetal or adult HSCs leads to the generation of multipotent hematopoietic HSC-like cell lines (Pinto do Ó 1998; Pinto do Ó 2002; Dahl 2008). Thus, Lhx2 has been shown to be an important regulator of stem and progenitor cell function and of mesenchymal-epithelial interactions during organ development.
Aim

The aim of this licentiate thesis is to elucidate the function of Lhx2 during hair formation. More specifically:

- To examine the expression pattern of Lhx2 during HF morphogenesis and the cyclic growth of the postnatal HF.
- To study the function of Lhx2 in these processes by investigating gain-of-function and loss-of-function mouse models.
Results and discussion

Expression of Lhx2 during HF morphogenesis and postnatal cycling

When our group had studied Lhx2 expression in diverse organs of the mouse we had often seen strong and consistent Lhx2 expression in the HFs when skin was present on the sections. That woke our interest since we previously had shown that Lhx2 is a potent regulator of stem cell function and critically involved in mesenchymal-epithelial interactions during development of various organs.

We found Lhx2 to be expressed already at stage 0 of HF morphogenesis prior to any morphological signs of hair placode formation and Lhx2 expression was subsequently detected in the hair placode, primary HG and early hair peg. When the IRS starts to form at stage 4, Lhx2 expression is turned off in these cells and becomes restricted to the ORS and matrix cells in the bulb and this expression pattern is maintained during stages 5 to 8 of HF morphogenesis (Figure 1A-E, Paper I). No Lhx2 expression was detected in telogen HFs (Figure 1J and 2C, Paper I). In late telogen, before any morphological signs of anagen induction and expression of the anagen-specific gene Shh, Lhx2 expression reappears in the 2°HG (Figure 2H-I, Paper I). During the first substages of anagen (I and II), Lhx2 mRNA was detected in the 2°HG and the future matrix cells surrounding the DP. When anagen progresses to substage IIIa, Lhx2 expression is turned off in the forming IRS cells (Figure 2L and P, Paper I). During later substages of anagen (IV-VI) Lhx2+ cells are present in a subpopulation of the matrix cells in the proximal part of the bulb and scattered in the ORS (Figure 2T-T’ and 1F-G, Paper I), and resembles the expression pattern during late HF morphogenesis (Figure 1E, Paper I). Few, if any, cells co-express Lhx2 and CD34, a marker of bulge stem cells (Figure 2F, K, O and S, Paper I).

The morphogenesis- and anagen-associated expression of Lhx2 indicated a role for Lhx2 in hair generation and regeneration. However, our results do not fit with those previously published by Rhee et al. (2006). They used immunohistochemistry to detect Lhx2 in developing hair placode, primary HG and peg. In the more mature HF and in adult mice, Rhee et al. claim that Lhx2 protein becomes restricted to cells in the bulge and they
claim the function of Lhx2 is to maintain the quiescent character of the bulge stem cells. We detected Lhx2 protein in the ORS and in the matrix cells in the hair bulb (Figure 1H-I, Paper I), in agreement with the mRNA expression. Lhx2 protein appears to persist after mRNA down-regulation during differentiation and distally movement of matrix cells, whereas the mRNA is enriched in the cells from where the matrix cells originate in the proximal part of the bulb (compare Figure 1H and 1G, Paper I). This observation suggests that Lhx2 protein persists longer than mRNA, which has been observed previously in the olfactory epithelium (Kolterud 2004a). Whether this observation can explain why we detected Lhx2 protein but no mRNA in the proximal part of the bulge (Figure 2K’, Paper I) remains to be elucidated.

When Rhee et al. use in situ hybridization to detect Lhx2 expression during HF morphogenesis, the results agree with the expression pattern detected by us. When they instead are using immunohistochemistry they preferentially detect Lhx2 protein in the leading edge of the developing HFs. That would be more in line with our interpretation of Lhx2 function, since the cells of the leading edge should correspond to matrix cells and be proliferating and differentiating. As soon as Rhee et al. study adult HFs they only use immunohistochemistry to detect Lhx2 protein and then their results differ from ours. However, I am confident in our results since they have been confirmed both on the mRNA and protein level, and we can show overview figures over several HFs with the same expression pattern (Figure S3, Paper I). Moreover, that Lhx2 expression is varying depending on phase in the hair cycle has also been observed when global gene expression was analyzed at different HF cycle stages (Lin 2009).

Transgenic over-expression of Lhx2 in HFs results in anagen induction

Our first experiments to investigate a possible function of Lhx2 in HF biology were to over-express Lhx2 in HFs. In order to do that we generated a Z/Lhx2-GFP mouse strain, which is transgenic for a DNA construct based on the double reporter vector Z/AP (Lobe 1999). A β-Geo gene (encoding a β-galactosidase-neomycin fusion protein) flanked by lox sites is followed by the Lhx2 cDNA, an internal ribosomal entry site (IRES) and the green
Figure 5: Vectors used for generation of mouse models. A) Schematic representation of the vector used to generate the Z/Lhx2-GFP transgenic mouse strain (upper panel) and the organisation of this vector after Cre-mediated recombination (lower panel). The white arrows correspond to the mRNA that is generated before and after Cre-mediated recombination of the vector. B) Description of the Lhx2\textsuperscript{flx} allele where lox sites are flanking exon 2. C) Description of the hypomorphic allele of Lhx2 (Lhx2\textsuperscript{Neo}) containing the Neo gene between exon 2 and 3 in the opposite transcriptional orientation. lox sites are indicated by black triangles.

Figure 6: β-Gal activity in the epidermis and ORS in Z/Lhx2-GFP mice during telogen (A) and anagen (B). In Z/Lhx2-GFP: Nestin-Cre mice the β-Gal activity was absent in skin (C).
fluorescent protein (GFP) cDNA (Figure 5A). The mouse strain showed β-Gal activity in the epidermis and in the epithelial portion of the HFs during both telogen and anagen (Figure 6A-B). Following Cre recombination, β-Geo is excised and the Lhx2-GFP part becomes placed directly downstream of the promoter leading to its transcription. Z/Lhx2-GFP mice were initially crossed to Nestin-Cre mice, where expression of Cre recombinase is regulated by a part of the nestin promoter which directs Cre expression to the progenitor cell population in HFs (Li 2003). All cells in the skin in Z/Lhx2-GFP:Nestin-Cre double transgenic mice were β-Gal− (Figure 6C) revealing that β-Geo had been replaced by the Lhx2-GFP part. Two Z/Lhx2-GFP:Nestin-Cre mice were analyzed at 15 and 19 weeks of age, respectively (unpublished data). The HFs in dorsal skin of the 15 week old mouse were in anagen (Figure 7A) whereas the control HFs were in telogen (Figure 7C). Also the HFs of the 19 week old Z/Lhx2-GFP:Nestin-Cre mouse were in anagen (Figure 7B) whereas the 19 week old control HFs were in telogen (Figure 7D). The HFs in both Z/Lhx2-GFP:Nestin-Cre mice expressed Lhx2 and part of the Lhx2 expression corresponded to transgenic Lhx2 since also GFP mRNA was detected (data not shown). These results suggest that Lhx2 is correlated to anagen which was in agreement with the expression analysis.

However, few Z/Lhx2-GFP:Nestin-Cre mice survived until they were older than one week (Table 1, upper panel) and we never obtained any additional adult double transgenic mice. The expected ratio is 25 % of each genotype but only 2.6 % were found to be positive for both transgenes. If instead embryos and newborn mice were genotyped, the distribution was the expected with 24 % double positive mice out of a total of 33 (Table 1, lower panel). Thus, most of the transgenic mice die during early postnatal life. We do not know if the pups die by themselves or if the mother kills them, but it might be that the double transgenic mice have some severe defect due to over-expression of Lhx2 in the brain (Figure 7E). The low viability resulted in that only two double transgenic mice were analyzed and the analysis was done at a late time point when the hair cycle is irregular and unpredictable. However, the results were still interesting and together with the expression pattern of Lhx2 it encouraged us to proceed with the gain-of-function studies using another mouse model.

We decided to instead breed the Z/Lhx2-GFP mice to transgenic mice expressing a fusion protein between Cre and the ligand binding domain of the Estrogen receptor (CreER). Application of tamoxifen
Figure 7: A-D) Hematoxylin-eosin stainings showing that HFs were in anagen in dorsal skin section from a 15 week old (A) and 19 week old (B; thick section) Z/Lhx2-GFP:Nestin-Cre mouse. HFs in control mice were in telogen at both 15 weeks (C) and 19 weeks (D) of age. E) In situ hybridization with a GFP probe on head from a Z/Lhx2-GFP:NestinCre embryo (E15.5) showing strong expression of GFP and thus transgenic Lhx2 in the forebrain.

(Tx) to the skin leads to nuclear translocation of the CreER protein (Hayashi 2002) and hence deletion of β-Geo and transcription of the Lhx2-GFP part of the DNA. The Z/Lhx2-GFP:CreER mice were shaved and Tx treated at the age of 5 weeks, during the first postnatal anagen phase. When analyzed at 9 weeks of age, when the majority of the control animals were in telogen, all analyzed Z/Lhx2-GFP:CreER mice (6/6) were in anagen (Figure 5A-B and Table 1, Paper I). These results can be interpreted in two different ways. Either the anagen phase is prolonged when Lhx2 is over-expressed, or the anagen phase is prematurely induced during telogen. A prolonged
Table 1: Genotypes of offspring from Z/Lhx2-GFP x Nestin-Cre breedings.

<table>
<thead>
<tr>
<th>Genotyped when &gt; 1 week old</th>
<th># genotyped mice</th>
<th>% of total</th>
<th>Expected %</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt:wt</td>
<td>67</td>
<td>34 %</td>
<td>25 %</td>
</tr>
<tr>
<td>Z/Lhx2-GFP:wt</td>
<td>58</td>
<td>30 %</td>
<td>25 %</td>
</tr>
<tr>
<td>Wt:Nestin-Cre</td>
<td>66</td>
<td>34 %</td>
<td>25 %</td>
</tr>
<tr>
<td>Z/Lhx2-GFP:Nestin-Cre</td>
<td>5</td>
<td>2.6 %</td>
<td>25 %</td>
</tr>
<tr>
<td><strong>Totally 196</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genotyped embryos &amp; newborn</th>
<th># genotyped mice</th>
<th>% of total</th>
<th>Expected %</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt:wt</td>
<td>8</td>
<td>24 %</td>
<td>25 %</td>
</tr>
<tr>
<td>Z/Lhx2-GFP:wt</td>
<td>10</td>
<td>21 %</td>
<td>25 %</td>
</tr>
<tr>
<td>Wt:Nestin-Cre</td>
<td>7</td>
<td>30 %</td>
<td>25 %</td>
</tr>
<tr>
<td>Z/Lhx2-GFP:Nestin-Cre</td>
<td>8</td>
<td>24 %</td>
<td>25 %</td>
</tr>
<tr>
<td><strong>Totally 33</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

anagen phase would result in an angora phenotype as seen in mice with deleted FGF5, which show noticeably longer hair when the anagen is extended with one week (Hébert 1994). We did not note an increase in hair length and thus we suspected that anagen could be prematurely initiated. To address this question, mice were analyzed at an earlier time point closer to the anagen-catagen-telogen transition. At this timepoint the HFs in one out of three Tx-treated Z/Lhx2-GFP:CreER mice were in telogen (Figure 5D-F and Table 1, Paper I), suggesting that HFs containing cells expressing transgenic Lhx2 can enter telogen but prematurely reenter anagen since HFs in all mice at 9 weeks of age were in anagen.

Since we could not distinguish transgenic Lhx2 from endogenous Lhx2 in the over-expressing mice, we analyzed GFP expression and β-Gal activity. Most of the HFs in Tx-treated Z/Lhx2-GFP:CreER mice showed GFP expression, and there was an enrichment of GFP+ cells in the proximal part of the hair bulb (Figure 5H, Paper I). This corresponded to an increase in β-Gal+ cells in the proximal part of the bulb compared to control HFs in Z/Lhx2-GFP single transgenic mice (Figure 5K-L, Paper I), since GFP expression and β-Gal activity is mutually exclusive in each cell. The distribution and number of GFP+ cells varied between HFs in Z/Lhx2-GFP:CreER mice, but GFP expression was easily detected in the ORS and in cells scattered in the IRS (Figure 5H-I, Paper I). Lhx2 was, as GFP,
expressed in the bulb, ORS and parts of the IRS (Figure 5M, Paper I). The Lhx2+ cells in the IRS suggest that HF progenitor cells can migrate and differentiate into IRS cells despite maintained expression of Lhx2. Lhx2 had a wider expression than GFP in the Tx-treated Z/Lhx2-GFP:CreER mice as it was expressed in all HFs and often in more cells in the HFs compared to GFP, showing that endogenous Lhx2 was also expressed as during normal anagen. Thus the cells expressing the Lhx2 transgene can induce endogenous Lhx2 expression in a cell nonautonomous manner.

Numerous β-Gal+ cells were present in the ORS after Tx-treatment of Z/Lhx2-GFP:CreER mice (Figure 5K, Paper I), showing that the excision of β-Gal is not complete. As a consequence, relatively few cells express the Lhx2 transgene in the HFs. The cells expressing transgenic Lhx2, recognized by their expression of GFP, were enriched in the proximal part of the hair bulb (Figure 5H, Paper I), where endogenous Lhx2 normally is expressed (Figure 1F-G, Paper I). Hence the cells expressing transgenic Lhx2 follow the same migration path as cells expressing the endogenous Lhx2. The transgenic Lhx2+ cells would thus be close to the DP and possibly transmit epidermal signals to it whereas the DP could signal back and turn on the endogenous Lhx2 expression, explaining how relatively few Lhx2+ cells can induce anagen. Several BMP inhibitors and FGFs, especially FGF7, are upregulated in the DP in late telogen HFs compared to early telogen HFs, and those genes are thought to be involved in the signaling from the DP at anagen onset (Greco 2009). It remains to be elucidated if Lhx2 upregulates the expression of these genes or vice versa.

**Mice with inactivated Lhx2 are unable to regenerate a normal hair shaft**

Since over-expression of Lhx2 in HFs prematurely induces anagen, we also wanted to elucidate the phenotype in HFs where Lhx2 had been inactivated. A mouse strain with lox sites flanking exon 2 of the Lhx2 gene was obtained (Figure 5B), providing an opportunity to conditionally inactivate Lhx2 by expression of the Cre recombinase. The allele was named Lhx2\textsuperscript{flox} and mice homozygous for this allele (Lhx2\textsuperscript{flox/flox}) developed normally. The Lhx2\textsuperscript{flox/flox} mice were bred into mice carrying the null allele of Lhx2 (Lhx2\textsuperscript{−}; Porter 1997) and CreER mice to generate CreER\textsuperscript{Lhx2\textsuperscript{flox/flox}}, CreER:Lhx2\textsuperscript{flox/flox},
Application of Tx to the skin leads to deletion of exon 2 and as the remaining mRNA becomes out-of-frame it results in a conditional inactivation of the Lhx2 gene in the epidermis of CreER:Lhx2^floxflox and CreER:Lhx2^flox/- mice. The Lhx2^floxflox and Lhx2^flox/- mice were unaffected by the Tx treatment and used as controls. The mice were shaved and treated with Tx on their back skin at the age of 3 weeks during their first telogen and analyzed during their first postnatal anagen phase which is initiated at approximately 4 weeks of age. All control animals (9/9) did regrow their hair on the shaved area while most of the Tx-treated CreER:Lhx2^floxflox and CreER:Lhx2^flox/- mice did not (6/8; Figure 3A, Paper I). Lhx2 mutant HFs initiate anagen (Figure 3D, Paper I) similar to control animals (Figure 3B, Paper I) and progress to anagen substage III but were unable to assemble a normal hair shaft (Figure 3E, Paper I). HFs outside of the treated area in these mice form hair shafts indistinguishable from control HFs (Figure 3F, Paper I). Two of the Tx-treated CreER:Lhx2^floxflox mice did re-grow some hair one week later than the controls due to an incomplete Lhx2 inactivation (see below). An additional set of mice, shaved and Tx-treated at the same time point, were analyzed at 9 weeks of age when most control animals had entered the second and extended telogen phase. One conditional mutant had not re-grown hair despite that it had dark skin which indicates that the HFs are in anagen (Müller-Röver 2001). All HFs did also contain proliferating cells positive for Hist1h3c expression, only expressed during anagen (Figure 3I and S6, Paper I). Although some HFs had formed hair shafts, the hair shafts appeared to be distorted and the HFs never developed beyond anagen substage III (Figure 3K, Paper I). Thus anagen progression is severely compromised in the mutated HFs.

To confirm that the Lhx2 gene was inactivated we performed in situ hybridizations using two different Lhx2 probes. The first was a probe from the full-length cDNA which hybridizes to mRNA expressed from both wildtype and inactivated allele (Kolterud 2004b; Monuki 2001). We also used an exon 2-specific probe that only hybridizes to the wildtype mRNA as exon 2 is deleted in both the conditionally inactivated Lhx2^floxflox allele and in the Lhx2^- allele. In the HFs with arrested hair shaft development cells expressed mRNA hybridizing to the full-length Lhx2 probe, as expected, but the mRNA did not hybridize to the exon 2-specific probe and thus there was no functional Lhx2 mRNA in these cells (Figure 3G and K, Paper I). In the Tx-treated CreER:Lhx2^floxflox mice that regrew their hair, cells expressing
mRNA hybridizing to both Lhx2 probes were present in the proximal part of the HFs (Figure S5, Paper I), showing that cells expressing the wildtype allele had reappeared. None of the CreER:Lhx2<sup>fl<sub>ox</sub></sup> mice regrew their hair, revealing that Lhx2 inactivation was more effective in these mice compared to animals with two Lhx2<sup>fl<sub>ox</sub></sup> alleles. That only a few cells retaining expression of wildtype Lhx2 could rescue hair formation further supports our hypothesis that Lhx2 is important for anagen progression.

**Significantly reduced levels of Lhx2 during morphogenesis results in decreased number of HFs and arrested or delayed development**

Rhee et al. (2006) observed a reduction in density of HFs in E16 Lhx2 null embryos compared to controls but were unable to analyze the further development because of the embryonic lethality of the Lhx2<sup>-/-</sup> mice. The embryos die at E15-17, prior to or at onset of pelage HF morphogenesis. To try to overcome this issue we obtained a hypomorphic allele of Lhx2. A Neo gene was placed between exon 2 and 3 in the Lhx2 gene in the opposite transcriptional orientation (Figure 5C), and the allele was denoted Lhx2<sup>Neo</sup>. Homozygous mice (Lhx2<sup>Neo/Neo</sup>) had significantly reduced levels of Lhx2 since embryos showed an eyeless phenotype similar to that of Lhx2<sup>-/-</sup> mice (Figure S4C, Paper I). Lhx2 expression was still detected revealing that Lhx2<sup>Neo</sup> was not a null allele (Figure 4D, Paper I). The embryos were also less affected by the anemia since the expected number of live Lhx2<sup>Neo/Neo</sup> embryos was obtained at E18.5. Thus, pelage HF formation could be studied up to a stage when it is well established. The number of formed HFs was compared between control and Lhx2<sup>Neo/Neo</sup> mice, and at E16.5 the HF density in hypomorphic embryos was approximately half of the density in controls, corresponding to the results obtained by Rhee et al. At E18.5 the density of HFs had increased considerably in wildtype embryos, whereas it had not increased in the skin of Lhx2<sup>Neo/Neo</sup> mice. Moreover, HFs appeared to have arrested prior to stage 5 in morphogenesis, before the formation of the IRS and hair shaft (Figure 4A-B, Paper I).
Figure 8: A, B) Alkaline phosphatase stainings of whisker HFs in Lhx2$^{+/+}$ and Lhx2$^{-/-}$ embryos at E15.5. The epidermal cells in the hair bulb are not enveloping the DP in Lhx2$^{-/-}$ whisker HFs (B) as in the control HFs (A).

We have not studied later time points since the Lhx2$^{Neo/Neo}$ animals appear to die shortly after birth and it is not clarified if the remaining HFs will eventually form. Lhx2 expression is initiated in basal epidermal cells prior to formation of the hair placode, supporting the hypothesis of an early function of Lhx2 in HF morphogenesis. It is also possible that Lhx2 has different function in different HFs depending on hair type. The regulation of tylotrich HFs differs from that of the non-tylotrich, and there can also be differences between awl, auchene and zigzag hairs. Loss of Lhx2 might be compensated for by other genes in specific hair types. In limb development Lhx2 can be compensated for by the LIM homeobox genes Lhx9 and Lmx1b (Tzchori 2009), but these genes are not expressed in skin (unpublished data). A future study is to compare Lhx2 function in separate hair types. We have studied the morphogenesis of whisker HFs in Lhx2$^{-/-}$ mice and found all expected whisker HFs to be formed (unpublished data). However, in contrast to in normal whiskers mutant epidermal cells fail to completely envelop the DP (Figure 8). Interestingly, the same phenotype is observed in the developing pelage HFs of the Lhx2$^{Neo/Neo}$ mice (Figure 4A-B, Paper I). The relevance of this finding remains to be elucidated but it could indicate that the interaction between the epidermis and the DP is distorted. Besides the diminished number of pelage HFs in skin with considerably reduced Lhx2 expression, the formed HFs appeared to have arrested before the formation of the IRS and hair shaft. Since we have not followed the HF morphogenesis further we cannot definitely rule out that the process is delayed.
**Lhx2 and the HF stem cells**

An important process to understand in the HF field is the cellular mechanisms governing the induction of the anagen phase. Already 40 years ago it was reported that proliferation is initiated in the 2°HG during late telogen and early anagen (Silver 1969; Silver 1970), however the controversial issue is the role of the 2°HG in this. According to the bulge activation hypothesis, anagen is initiated when bulge stem cells start to proliferate upon receiving an activation signal from the DP, and the forming transient amplifying cells migrate to the 2°HG to proliferate and differentiate into all layers of the developing HF (Sun 1991). The HF predetermination hypothesis suggests that the 2°HG itself contains stem cells which start to proliferate upon signaling from the DP without input from the bulge (Panteleyev 2001). The bulge activation hypothesis has been the dominating theory mainly because of that the bulge contain label-retaining cells whereas the 2°HG does not (Cotsarelis 1990). However, lately there have been an accumulation of reports that support the HF predetermination theory and give the 2°HG a more significant function. Ito et al. (2004) have proposed the 2°HG cells to be able to originate the regrowth of the lower transient part of the HF during anagen progression and also to repopulate the bulge stem cell niche after injury, implying the 2°HG cells to contain self-renewing stem cells. Greco et al. (2009) showed the 2°HG cells do respond quicker than bulge stem cells to the signals from the DP at anagen onset. Already during late telogen, 2°HG cells have entered the cell cycle whereas the bulge stem cells are quiescent until a later stage during anagen. The 2°HG cells resemble activated bulge stem cells molecularly but are not label-retaining (Greco 2009). Our results suggest that Lhx2 is expressed by the activated 2°HG stem cells during late telogen phase, and this is in agreement with our observation that over-expression of Lhx2 in the epidermal part of the HF is sufficient to prematurely induce anagen. Presumably the transgenic Lhx2 expression leads to a premature activation of the 2°HG cells. An interesting experiment would be to construct a mouse strain with strong transgenic expression of Lhx2 after the 2°HG specific P-cadherin promoter (Greco 2009). A believable outcome is that the 2°HG stem cells becomes constantly active and telogen is shortened even more and/or anagen is extended.

The leucine-rich G protein-coupled receptor 5 (Lgr5) has been identified as a marker for a proliferating HF stem cell population distinct from the quiescent stem cell pool in the bulge (Jaks 2008). Although
the Lgr5\textsuperscript{+} cells are cycling, they are long-lived and have the potential to give rise to all layers of the growing HF during anagen and can also contribute to the permanent part of the HF up to the level of the SG including the bulge and thus are (or contain) multipotent stem cells (Jaks 2008). When comparing their reported expression pattern of Lgr5 with the Lhx2 expression presented in this work, there are explicit similarities (Figure 9A-B). Lgr5 is however expressed during the whole telogen phase and in the proximal part of the bulge in addition to in the 2°HG. Lgr5 expression overlaps with CD34\textsuperscript{+} cells in the proximal part of the bulge (Figure 9C). When Lhx2 expression appears just prior to anagen induction it seems to be in Lgr5\textsuperscript{+} cells in the 2°HG, and during anagen both Lhx2 and Lgr5 are expressed in the ORS and proximal part of the hair bulb. We have to confirm that the expression is in the same cells, but supporting that is a real-time PCR analysis showing Lhx2 to be exclusively expressed by Lgr5\textsuperscript{+} cells (Jaks 2008). The results from the Lgr5 study as well as from our investigations of the expression and function of Lhx2 in HFs are consistent with the hypothesis of two coexisting stem cell populations, and Lhx2 appears to be a marker of activated 2°HG stem cells. The location of the 2°HG is easily defined during telogen as the part of the HF separating the bulge from the DP. However, during anagen it is more difficult to define. It has been

**Figure 9:** The expression pattern of Lhx2 (A) compared to the Lgr5 (B) and CD34 (C) expression during late telogen. Adopted from Jaks (2010).
suggested that the $2^\circ$HG cells are located just below the bulge also during anagen (Li 2010). It would be interesting to further study this to investigate the possibility that the stem cells of the $2^\circ$HG becomes scattered in the whole cycling part of the ORS and thus could correspond to the Lhx2$^+$ cells. Supportive of this is the suggestion of Jaks et al. (2008) that stemness is an intrinsic property of HF stem cells and does not depend on a specific niche.

Our results reveal an alternative interpretation of Lhx2 function compared to previously published results (Rhee 2006). Rhee et al. suggested Lhx2 to be important for maintaining the quiescent stem cell character of HF stem cells in the bulge whereas we have found Lhx2 to promote differentiation and patterning of HFs. Rhee et al. performed both gain-of-function and loss-of-function studies to analyze the function of Lhx2. They generated transgenic mice with $Lhx2$ expression under control of the K14 promoter leading to epidermal $Lhx2$ expression early during HF morphogenesis, and saw an induction of markers of adult HF stem cells in the embryonic skin. However, the number of formed HFs did not change. We instead studied the effect of transgenic $Lhx2$ expression in adult HFs in telogen, when there is no endogenous $Lhx2$ expression. During HF morphogenesis there is already wide expression of endogenous $Lhx2$. In the $Lhx2^{+/\text{lox}}$ (and $Lhx2^\text{Neo/Neo}$) embryos both we and Rhee et al. detected a diminished HF density compared to controls, which is in line with our observation that Lhx2 expression appears prior to the formation of a hair placode and thus possibly is involved in induction of HF morphogenesis. Rhee et al. engrafted Lhx2 null skin onto nude recipient mice and followed the cycling of the HFs for several weeks, and noticed a shortened telogen phase in the $Lhx2^{-/-}$ HFs compared to control HFs. The different theories about Lhx2 function could be explained by the different approaches used to study gain-of-function and loss-of-function. Our interpretation of the function of Lhx2 in the HFs is also in agreement with Lhx2 function in other tissues. Lhx2 is required for patterning of the telencephalon during brain development (Bulchand 2001; Monuki 2001) and for differentiation of progenitors into mature olfactory sensory neurons (Kolterud 2004a), and is involved in the expansion, differentiation and organization of the cellular components of the fetal liver (Kolterud 2004b; Wandzioch 2004).
**How does Lhx2 function in the HF**s?

We have not yet elucidated the molecular basis of Lhx2 function in the HF. In the Lhx2 gain-of-function experiments, Lhx2 appears to act upstream of Wnt and Shh signaling since over-expression of *Lhx2* can initiate those pathways and prematurely induce anagen. On the other hand, Lhx2 seems to act downstream of such pathways in the loss-of-function studies since central mediators of Hedgehog (Shh, Smo and Ptc1), BMP (BMP4 and noggin) and Wnt (Lef1 and β-catenin) signaling are present in the Lhx2 mutants (Figure 3J and 4D, Paper I). In the adult mice with inactivated *Lhx2* expression, this however could be a consequence of that *Lhx2* might not be completely inactivated in all HF before anagen is induced since the Tx-treatment proceeds during one week. The skin in mice homozygous for the hypomorphic *Lhx2*Neom allele (Figure 4D, Paper I) contained fewer and smaller follicles and that caused a problem to determine if the HF are fewer because of downregulated expression of the analyzed genes or that the genes are less expressed because there are fewer HF. However, all analyzed signaling molecules are present although it is difficult to quantify them.

To identify the signaling pathway(s) involved in Lhx2 function, one way is to study possible connections to other genes known to be important in hair shaft regulation. The co-factor of LIM domains 2 (*Clim2*) is expressed in the ORS and hair matrix and mice with transgenic expression of a dominant negative *Clim* regulated by the K14 promoter develop a progressive hair loss as the HF becomes incapable of producing hair shafts (Xu 2007). The phenotype indicates a function of Clim proteins in hair differentiation. Clim2 has been shown to interact with Lhx2 (Xu 2007) and the role of Lhx2 in hair formation is perhaps depending on its co-factor. Several genes involved in the Wnt pathway were downregulated in the Clim loss-of-function mice (Xu 2007). Furthermore, Msx1 is a transcription factor with a connection to Lhx2 since the HDs of Lhx2 and Msx1 mediate interactions between the two proteins, forming a protein complex in the absence of DNA (Bendall 1998). The function of this is unknown, but the complementary expression patterns of *Lhx2* and *Msx1* during embryogenesis in most tissues, e.g. the olfactory organ and neural tube (Lu 2000), suggest that it is important. The genes are partially co-expressed in the developing limb (Lu 2000) and in the adult HF. The expression of *Msx1* is concentrated to the matrix cells during anagen and is turned off when catagen begins (Ma 2003). The Msx family member *Msx2* is
expressed during early anagen in the matrix and during late anagen expression is expanded to the IRS, and expression is in contrast to Msx1 continued in catagen (Ma 2003). The Msx genes are important already during HF morphogenesis, as the double knockout (Msx1<sup>-/-</sup>;Msx2<sup>-/-</sup>) develop fewer HFs; at E18.5 they are reduced to approximately one-third of the number in controls (Satokata 2000). Msx2<sup>-/-</sup> mice display cycles of hair loss and regrowth and structurally deformed hair shafts (Ma 2003). The function of Msx proteins both during HF morphogenesis and in differentiation during anagen, resembling the double functions of Lhx2, suggests a connection between Lhx2 and the Msx proteins in HF biology. Msx2 as well as Foxn1 are found to function downstream of BMP signaling, and Msx2 and Foxn1 are together required to maintain Notch1 expression in the matrix, which in turn is necessary for the differentiation of IRS and the medulla of the hair shaft (Cai 2009; Lin 2000). A future experiment is to investigate the expression of hair shaft regulatory genes in our mice with conditionally inactivated Lhx2. If Lhx2 is positioned downstream of Wnt, Shh and/or BMP signaling no change would be seen in the expression of the mediators of those pathways. However, a diminished Lhx2 expression might influence the levels of Msx1, Msx2, Foxn1 and Notch1 (discussed above) if Lhx2 is positioned upstream of those genes. Other genes that are important for IRS differentiation and whose inactivation in HFs causes a phenotype similar to that when Lhx2 is inactivated, and thus could be regulated down-stream of Lhx2, is the Notch target Gata3 (Amsen 2007; Kaufman 2003) and Dlx3, which in turn functions upstream of Hoxc13 and Gata3 (Hwang 2008).

**Final conclusions**

We have shown that Lhx2 plays an important role in hair generation and regeneration. During HF morphogenesis Lhx2 has a widespread expression in the epidermal portion of the HF and decreased levels of the protein results in fewer HFs that in addition are arrested or delayed in development. Thus, Lhx2 is essential for HF morphogenesis. In adult mice Lhx2 has a cyclic expression pattern which is suggesting a role in hair cycle regulation, and the loss-of-function experiment also reveal that Lhx2 is required for postnatal hair formation. Furthermore, transgenic expression of Lhx2 in HFs results in premature anagen initiation and thus Lhx2 expression is sufficient to induce
anagen. Taken together, Lhx2 plays a role in differentiation and patterning of HFs.

The function of Lhx2 appears to be conserved between HF morphogenesis and anagen. The two processes resemble each other both morphologically and molecularly, including the expression pattern of Lhx2. Lhx2 is expressed prior to the formation of a placode during morphogenesis and before any visible signs of anagen induction during late telogen, suggesting a role of Lhx2 in initiation of HF morphogenesis and anagen. Furthermore, the arrest in HF morphogenesis in embryos with significantly reduced levels of Lhx2, and in anagen progression in mice with inactivated Lhx2 is at similar developmental stages before formation of the hair. Lhx2 has a complex role in HF biology as it is involved both in the initiation and progression of the HF morphogenesis and anagen.

It is important to study the HF and its regulation, partly because of potential clinical implications in hair disorders and skin injuries such as severe burns. The HF is also interesting as an autonomous mini-organ for studying adult stem cells and their function in organ regeneration. Elucidating the process of HF regeneration during the hair cycle could provide new insights to regenerative medicine of other organs. Our studies of Lhx2 contribute to the knowledge of how hair is generated and regenerated.
Enkel svensk sammanfattning

Hår är viktigt för temperaturreglering, fysiskt skydd, sensorisk aktivitet, säsongsstyrt kamouflage och sociala interaktioner. Varje hårstrå produceras av en hårsäck och vi människor har ungefär fem miljoner av dessa små organ i huden varav 80 000 till 150 000 sitter på skalpen. Det bildas inga nya hårsäckar efter födseln, men de som redan finns kan ändra karaktär. Till exempel under puberteten för pojkar omvandlas hårsäckarna som tillverkar de tunna, ljusa håren på hakan till att göra tjocka skägghårstrån, och senare i livet kan hårsäckar för långa hårstrån på skalpen börja göra små, ljusa hår. Hårsäckarna går kontinuerligt genom stadier av tillbakagång, vila och tillväxt under hela livet. Hur länge tillväxtfasen varar påverkar hur långt hårstrået blir, och det varierar mellan olika delar av kroppen. Hårsäckarna på skalpen har en tillväxtfas på 2 till 6 år emedan den endast är några månader för ögonbrynen. Det är viktigt att studera hårsäckarna, dels på grund av möjliga kliniska tillämpningar vid sjukdomar som rör hårväxten och vid skador på huden, t.ex. brännskador. Hårsäcken är också ett bra organ för att studera grundläggande principer inom biologi eftersom det är väldefinierat, lättillgängligt och kontinuerligt återbildas med hjälp av omogna stamceller. Det är inte klargott vilka gener som reglerar hur hårsäckarna bildas i embryot och hur snabbt cyklerna i vuxna hårsäckar ska gå. För att studera det kan man dels undersöka vilka gener som reglerar hur hårsäckarna bildas i embryot och dels utveckla möss som uttrycker antingen för mycket av genen eller där genen är helt avstängd för att se hur det påverkar hårsäckarna. Transkriptionsfaktorn Lhx2 har tidigare rapporterats viktig för utvecklingen av flera olika vävnader, bl.a. hjärna, öga och lever, och därför har vi undersökt dess funktion även i hårsäckarna.

Vi fann att Lhx2 börjar uttryckas i huden precis innan hårsäckarna bildas i embryot, och i vuxna alldeles i slutet av vilofasen innan en ny tillväxtfas börjar. Sedan är genen uttryckt under hela tillväxtfasen för att försvinna under vilofasen. I kontrast till tidigare publicerade forskningsresultat av andra forskargrupper verkar Lhx2 vara uttryckt av omogna celler utanför den region av hårsäcken där hårsäckens stamceller återfinns. När vi uttryckte Lhx2 under vilofasen när det normalt inte uttrycks något Lhx2 i hårsäckarna, startade tillväxtfasen tidigare än i kontrollmössen. När vi tvärtom inaktiverade genen i hårsäckarna på vuxna möss, fick inte mössen tillbaka håret på den del av ryggen som rakats och behandlats.
Tillväxtfasen inleddes men avstannade innan ett nytt hårstrå hade bildats. Vi prövade också att minska nivån av Lhx2 under embryoutvecklingen och då bildades färre hårsäckar och de som ändå bildades stannade i sin utveckling innan de började tillverka ett hårstrå. Alltså har vi funnit att Lhx2 är viktigt för bildningen och återbildningen av hår.
Acknowledgements

Först och främst vill jag tacka Leif för att jag fått vara här så många år och hålla på med något jag tyckt varit meningsfullt och intressant. Även om det i slutändan inte riktigt räckte ända fram till en doktorshatt, har jag uppskattat tiden här och de engagerande diskussioner med dig som blev tilltagande fler med åren.


Tack också till övriga och tidigare medlemmar av gruppen: Karin, Eva, Mona, Jörgen, Åsa och inte minst Ewa och Christofer som började med härprojektet en gång i tiden, och till resten av UCMM för hjälp med allt möjligt, fikasällskap och för trevliga ölhörnor.


Älskade Vega och Ester, ni är det bästa som någonsin hänt mig och ger mig energi att klara vad som helst. Tack för att ni finns!

Simon, utan dig hade det aldrig blivit någon bok. Tack för att du ställer upp, peppar och är världens bästa make, och jag ser så mycket fram emot att äntligen få mer tid till att vara med dig. And no more shall we part.
References


Kolterud Å, Wandzioch E, Carlsson L (2004b) Lhx2 is expressed in the septum transversum mesenchyme that becomes an integral part of the liver and the formation of these cells is independent of functional Lhx2. Gene Expr Patterns. 4:521-8.


Silver AF, Chase HB (1970) DNA synthesis in the adult hair germ during dormancy (telogen) and activation (early anagen). Dev Biol. 21:440-51.


