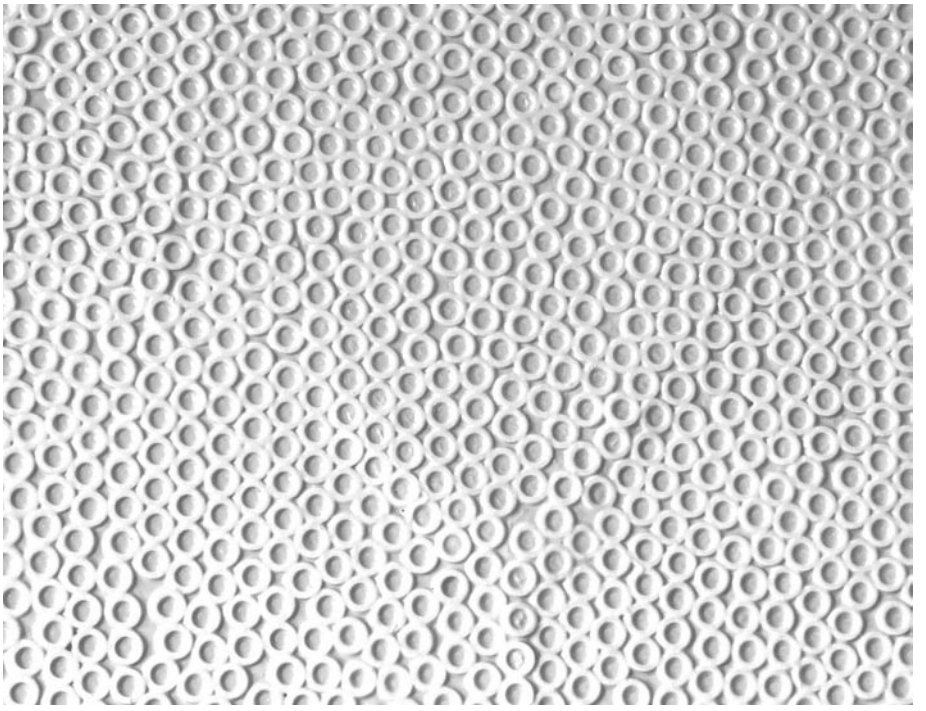
Studies on the role of Gata3 during development



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Studies on the Role of Gata3 During Development

De rol van Gata3 gedurende de ontwikkeling

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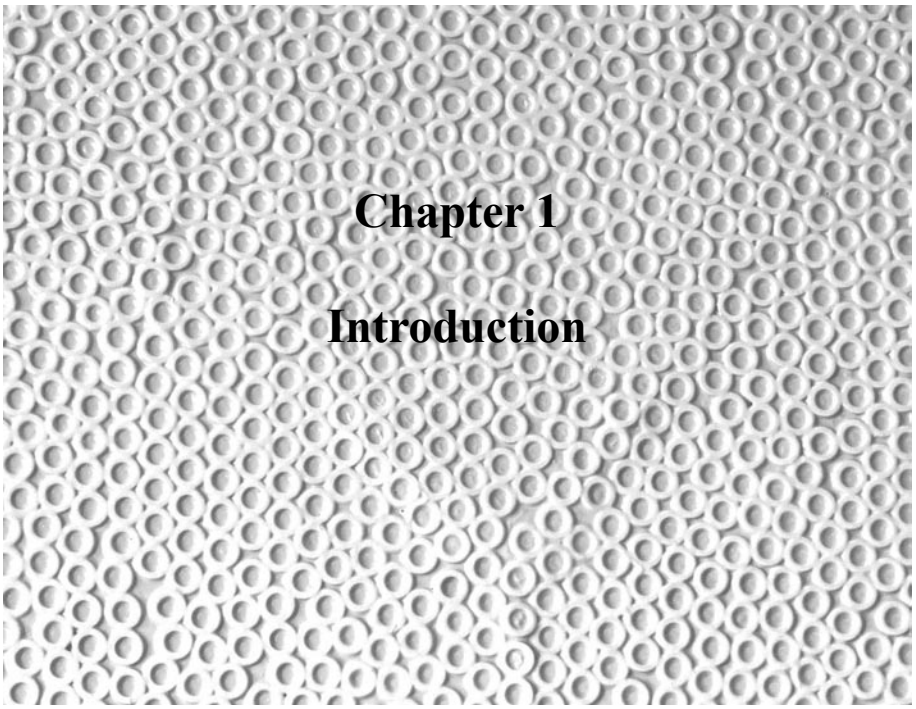
CENTRAAL STATION
"THE STORY STARTS WHEN.."
"..DOROTA COMES TO ROTTERDAM"
HI, MY NAME IS DOROTA & I'M LOOKING FOR A JOB!
I'm Marike. I'll introduce you to some people.
I'M JACQUELINE, & I WANT YOU TO WORK WITH Gata 3.
OK.
WHAT IS THAT GATA 3.?!
BANANAS! YOU ARE HIRED!
OOH! THANK YOU, PROF. GROSVELD!!
I'M Filipe. You can seat next to me.
THANKS!
So... MAYBE YOU CAN TELL ME.. WHAT IS GATA3?
No idea! You have to find out yourself.



Chapter 1

Introduction

Almost all cells in the living organism have identical genetic information, but each cell type expresses a limited number of genes. This phenomenon is regulated by specific proteins: transcription factors. These factors have the ability to recognize target sequences located at specific regulatory fragments located in or surrounding the gene (promoter, enhancer or LCR) and activate or repress gene transcription. Like many genes, the expression of transcription factors can also be restricted to particular cell types. This thesis is focused on the transcription factor GATA3, which is expressed in tissue specific manner during development. This chapter will introduce this factor and describes its sites of expression with the main focus on skin.

GATA factors

The mammalian GATA family of transcription factors is composed of six members, GATA1 to GATA6, which share related zinc-finger motifs with the sequence Cys-X2-Cys-X17-Cys-X2-Cys (where X represents any amino acid residue) and bind to the consensus motif 5'-(A/T)GATA(A/G)-3' (Ko and Engel, 1993; Ko et al., 1991; Merika and Orkin, 1993; Orkin, 1992). Outside the Zn-finger domains, conservation between the different GATA factors is poor and the homology of an individual member is higher between species than between different members of the same species (Orkin, 1992; Yamamoto et al., 1990).

The GATA family is divided into two subgroups based on the expression patterns of the individual transcription factors. GATA1, GATA2 and GATA3 are defined as the hematopoietic members of the GATA family as all three are expressed in hematopoietic system (Ohneda and Yamamoto, 2002; Patient and McGhee, 2002), although GATA2 and GATA3 are also widely expressed in other tissues. The non-hematopoietic subfamily is composed of GATA4, GATA5 and GATA6. They are expressed in tissues such as intestine, heart and lung but not in hematopoietic cells (Molkentin, 2000; Pikkarainen et al., 2004).

Gata3 is a member of the Gata transcription factor family

GATA3 belongs to the hematopoietic subfamily of GATA zinc finger transcription factors. It possesses N-terminal transactivation domains and two zinc fingers, the N-terminal zinc finger and the C-terminal zinc finger. The C-terminal zinc finger is essential for DNA binding, whereas the N-terminal zinc finger stabilizes this binding and physically interacts with other proteins such as the Friends of GATA (FOG) (Fox et al., 1998; Svensson et al., 1999; Takemoto et al., 2000; Tevosian et al., 1999; Tsang et al., 1997). The C-terminal region of GATA3 is highly conserved among the GATA family proteins. The amino acid motif (YxKxHxxxRP), adjacent to C-terminal zinc finger domain of GATA3, is important for GATA3 DNA binding and GATA3 functions, including the transcriptional activity and the ability to induce chromatin remodeling of the T-helper cell type 2 (Th2) cytokine gene loci leading to Th2 cell differentiation (Shinnakasu et al., 2006).

GATA3, which is expressed in different tissues to GATA1, can compensate for the GATA1 defect in ES cells (Blobel et al., 1995). Knock-in mutant embryos of the GATA1 locus in which GATA-3 cDNA was introduced by gene targeting exhibit partial rescue, characterized by increased survival of erythroid precursor cells and improved hemoglobin production (Tsai et al., 1998). Gata3, when expressed under the control of Gata1 regulatory sequences, is able to rescue the lethal anemia of the G1.05 knockdown mutation (Takahashi et al., 2000) and Gata3 transgene can also rescue the phenotype of the complete Gata1-null mutation (Ferreira et al., 2007).

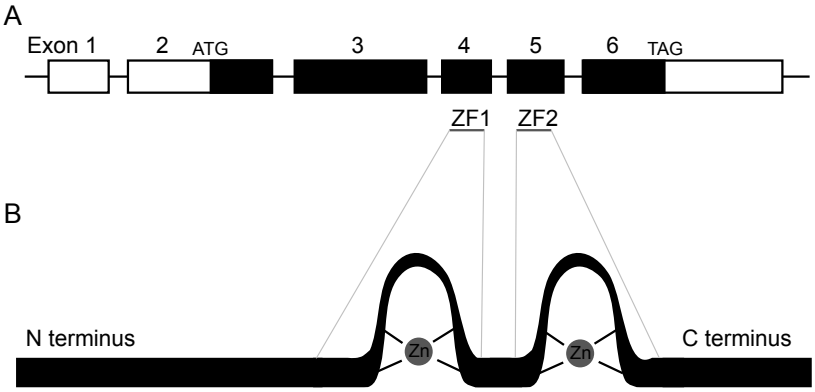


Figure 1. Gata3 gene and protein structure. (A) The genomic organization of the Gata3 gene comprises six exons. The ATG (start) site of translation is in exon 2 and the TAG (stop) codon is in exon 6. (B) The Gata3 protein domain structure. Its two zinc fingers (ZF1 and ZF2) and N terminus and C terminus are indicated.

Gata3 expression

Gata3 expression is temporally and spatially regulated during early murine development. Found most abundantly in the placenta prior to 10 days of embryogenesis, Gata3 expression becomes restricted to specific cells within the embryonic central nervous system (in the mesencephalon, diencephalon, pons and inner ear) later in gestation. Gata3 also shows a restricted expression pattern in the peripheral nervous system, including terminally differentiating cells in the cranial and sympathetic ganglia. In addition to this distinct pattern in the nervous system, Gata3 is also expressed in the developing parathyroids, inner ear, the eye, skin, mammary glands, embryonic kidney and the thymic rudiment (George et al., 1994; Labastie et al., 1995).

Gata3 is involved in the development of serotonergic neurons in the caudal raphe nuclei (Pattyn et al., 2004; van Doorninck et al., 1999), in ear formation (Karis et al., 2001; Lawoko-Kerali et al., 2002; Lillevali et al., 2006; Van der Wees et al., 2004), in sympathetic neuron development (Tsarovina et al., 2004), in mammary gland formation (Asselin-Labat et al., 2007; Kouros-Mehr et al., 2006), in epidermis and hair follicle development (this thesis, (Kaufman et al., 2003; Kurek et al., 2007) and in development of nephric ducts of the embryonic kidneys (Grote et al., 2006).
Targeted disruption of the Gata3 gene causes severe abnormalities in the nervous system and in fetal liver haematopoiesis (Pandolfi et al., 1995). Loss of GATA3 leads to deficits in tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH) expression, which results in reduced noradrenaline in the sympathetic nervous system and embryonic lethality. Gata3 mutants die at embryonic day 11 and they can be rescued to perinatality by feeding heterozygous intercrossed dams with catecholamine intermediates (Lim et al., 2000), with noradrenergic agonists (this thesis, (Pattyn et al., 2000) and by restoring Gata3 function specifically in sympathoadrenal lineages using human dopamine ß-hydroxylase (hDBH) gene promoter to direct GATA3 transgenic expression (Moriguchi et al., 2006).

Haploinsufficiency for GATA3 has been implicated in hypoparathyroidism, sensorineural deafness, renal anomaly (HDR) syndrome in humans (Van Esch et al., 2000). Mice heterozygous for GATA3 do not show any obvious renal abnormalities or hypoparathyroidism, however, heterozygous GATA3 ko mice suffer from hearing loss (van der Wees et al., 2004).

T-cells

T-cell development

After antigenic stimulation, naïve $CD4^+$ T cells in immunocompetent animals differentiate toward T helper type 1 (Th1) or Th2 cells when stimulated through their T cell receptors by peptide presented by specialized antigen-presenting cells (APCs) (Murphy and Reiner, 2002 and Figure 2). Establishment of these two lineages of Th cells can be influenced by variety of factors, including genetic background, type of antigen and route of administration, mode of presentation, degree of costimulation, and the cytokine milieu (reviewed by Glimcher and Murphy, 2000; Murphy et al., 2000; O'Garra and Arai, 2000). Th1 cells produce interferon-γ (IFN- γ), interleukin 2 (IL-2) and lymphotoxin and are important for protection against intracellular pathogens (cellular immune response). Inappropriate Th1 responses have been implicated in inflammatory and autoimmune diseases (Liblau et al., 1995; O'Garra et al., 1998; O'Garra et al., 1997; Seder and Paul, 1994). Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13 and are involved in humoral responses for host defense during parasitic infections and allergic reaction. Interleukins produced by Th2 cells are required for IgE production and activate mast cells and eosinophils, and enhanced activation of those cytokines involved in regulation of IgE leading to allergic inflammation.

T-cell Gata3 expression

GATA3 is abundantly expressed in T lymphocytes (Yamamoto et al., 1990);(George et al., 1994), throughout thymocyte development (Hendriks et al., 1999) and its expression is required for the development of T cells in the thymus (Hattori et al., 1996; Ting et al., 1996); and for β-selection and single positive CD4 thymocyte development (Pai et al., 2004). GATA3 appears to be a key factor for Th2 cell differentiation (Zhang et al., 1997; Zheng and Flavell, 1997), as is T box transcription factor T-bet for Th1 differentiation (Szabo et al., 2002). Gata3 is expressed in naïve T cells and strongly upregulated during Th2 development but subsides to a low level during in vitro Th1 differentiation (Zheng and Flavell, 1997).

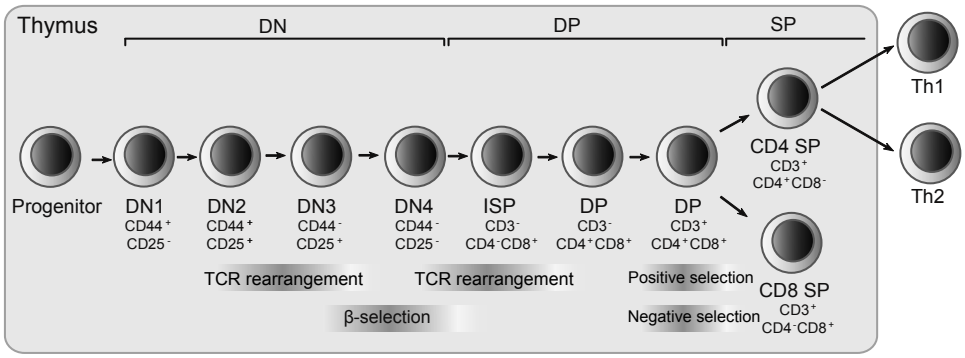


Figure 2. T-cell differentiation. The thymus is colonized by bone marrow-derived progenitors which are CD4 CD8 double negative (DN) 1 stage. Precursors become restricted to the T-cell lineage at DN2 stage and by the DN3 stage, the T-cell receptor β chain (TCRβ) locus starts to be rearranged. Cells that have successfully undergone β-selection mature via an immature single positive stage (ISP $CD8^+$) and start expressing CD4 and CD8 antigens while entering the double positive (DP) stage. At this stage, the TCRα locus is rearranged followed by positive and negative selection of TCRαβ presented at the surface. Single positive (SP) T cells that express CD4 or CD8 antigen and a functional TCR exit the thymus. Upon the stimulation naïve $CD8^+$ cells differentiate into cytotoxic T-cell and naïve $CD4^+$ T-cells differentiate into T-helper cell type 1 (Th1) or Th2.

GATA3(-/-) ES cells can contribute to the development of the mature erythroid, myelomonocytic and B-cell lineages, but fail to give rise to thymocytes or mature peripheral T cells. GATA-3(-/-) ES cells are unable to contribute measurably to the double-negative (CD4-/CD8-) thymocyte population, suggesting that differentiation of GATA-3(-/-) T cells is blocked at or before the earliest double-negative stage of thymocyte development (Hendriks et al., 1999; Ting et al., 1996).

Ectopic expression of Gata-3 induces Th2-specific cytokine expression not only in developing Th1 cells, but also in otherwise irreversibly committed Th1 cells and Th1 clones (Lee, H. J. et al., 2000).

Conditional deletion of the Gata3 gene at the DN stage using the Cre-loxP system, whereby the Cre transgene was driven by the proximal Lck promoter,

resulted in a developmental arrest at the $CD25^{+}CD44^{-}$ DN3 stage, implicating GATA3 in β-selection (Pai, S. Y.2004). Deletion of the Gata3 gene after β-selection, using CD4-Cre transgenic mice, resulted in increased CD5 levels at DP, CD4 SP and CD8 SP subpopulations and in a profound specific deficiency of CD4 SP cells (Ling et al., 2007; Pai et al., 2004). These findings demonstrated the absolute requirement of GATA3 for survival or development of CD4-committed thymocytes in vivo (Pai et al., 2004). Conversely, enforced GATA3 expression resulted in significantly reduced CD5 expression in DP cells, irrespective of their commitment towards the CD4 or CD8 lineage and sustained overexpression of GATA3 in fetal thymic organ cultures favored selection of CD4 over CD8 SP cells, but did not divert major histocompatibility complex (MHC) class I-restricted precursors into the CD4 lineage (Hernandez-Hoyos et al., 2003). On the basis of these findings it has been concluded that GATA3 is necessary for post-commitment CD4 generation, rather than for commitment to the CD4 lineage (Hernandez-Hoyos et al., 2003; Pai et al., 2004). Nevertheless, a possibility remains that GATA3 promotes the CD4 lineage choice, as it is conceivable that MHC class II-restricted $CD8^{+}$ T cells or MHC class I-restricted $CD4^{+}$ T cells die as they fail to undergo MHC-TCR and CD4/CD8 co-engagement required for their survival (Bosselut, 2004).

Regulation of and regulation by Gata3

GATA3 binding motifs have been identified in the promoters of the IL-5 and IL-13 genes (Kishikawa et al., 2001; Lavenu-Bombled et al., 2002; Lee, H. J. et al., 1998; Siegel et al., 1995), and GATA3 regulates transcription of IL-5 and IL-13 genes (Lee, H. J. et al., 1998; Schwenger et al., 2001; Zhang et al., 1998; Zheng and Flavell, 1997). In addition to the promoter regions, GATA3 also binds to various regulatory regions for Th2 cytokine expression, including the conserved GATA3 response element (CGRE) (Yamashita et al., 2002), the 3' site of IL-4 (VA) (Agarwal et al., 2000), the IL-4-IL-13 intergenic region (CNS1)(Takemoto et al., 2000), and the 3' end of RAD50 gene (Fields et al., 2004).

Changes in the chromatin structure of the Th1 cytokine (IFN-γ) and the Th2 cytokine (IL-5/IL-4/IL-13) gene loci occur during Th1/Th2 cell differentiation (Ansel et al., 2006; Lee, G. R. et al., 2006; Lohning et al., 2002). The histone modifications of the Th2 cytokine gene loci are primarily mediated through GATA3 (Inami et al., 2004; Omori et al., 2003; Yamashita et al., 2002). The binding of GATA3 at the CGRE region appears to initiate the long-range histone hyperacetylation accompanied by intergenic transcription within the IL-13/IL-4 gene loci in developing Th2 and Tc2 cells (Omori et al., 2003; Yamashita et al., 2002).

Transforming growth factor beta (TGF-β) is an important immunomodulatory cytokine that can inhibit the differentiation of CD4(+) cells into Th2 cells. Inhibition of GATA-3 expression by TGF-β is a major mechanism for Th2 differentiation, ectopic expression of GATA-3 in developing T cells overcomes the ability of TGF-β to inhibit

Th2 differentiation. TGF-β likely inhibits GATA-3 expression at the transcriptional level and does so without interfering with IL-4 signaling (Gorelik et al., 2000).

GATA-3 is activated in response to Notch-Delta signaling as an early and essential event in T lineage specification (Amsen et al., 2007; Fang et al., 2007). GATA-3, when highly expressed, diverts thymocytes from the T lymphoid pathway by activating a mast cell–like program independently of the exogenous mast cell growth factors IL-3 and SCF. Diversion occurred at the same stages of pro–T cell development as those at which GATA-3 is normally required and was highly efficient, especially for DN2 thymocytes. Thus, the positive functions of GATA3 for T lineage development are intrinsically dose dependent (Taghon et al., 2007).

Placenta

GATA3 together with GATA2 is expressed in placental trophoblast giant cells and was found to regulate transcription of the placental lactogen I gene, as well as the related proliferin gene (Steger et al., 1994)

Ear

Gata3 is involved in axonal navigation of the inner ear efferent neurons and, simultaneously, in the morphogenesis of the inner ear. GATA-3 null mutant mice show unusual axonal projections, such as misrouted crossing fibers and fibers in the facial nerve. GATA-3 is expressed both in the inner ear and in afferent and efferent auditory neurons. Specifically, GATA-3 is expressed in a population of neurons in rhombomere 4 (r4) that extend their axons across the floor plate of r4 at embryonic day 10 (E10) and reach the sensory epithelia of the ear by E13.5. In the ear, GATA-3 is expressed inside the otocyst and the surrounding periotic mesenchyme. In the inner ear, GATA3 is expressed in virtually all cell types that occur during ontogenesis including inner hair cells (IHCs), outer hair cells (OHCs), and various supporting cells (Debacker et al., 1999; Karis et al., 2001; Lawoko-Kerali et al., 2002; Rivolta and Holley, 1998).

Gata3 deficient embryos suffer from a severe arrest in inner ear morphogenesis. Ears of GATA-3 null mutants remain cystic, with a single extension of the endolymphatic duct. No semicircular ducts form and the cochlear ducts remain very short (Karis et al., 2001). Fibroblast growth factor 10 (FGF10) was identified as a downstream target of Gata3 in the ear (Lillevali et al., 2006). Gata3 is thought to participate also in hair cell differentiation since it is expressed in most inner ear sensory epithelia during development (Lillevali et al., 2004).

GATA3 haploinsufficiency is known to cause hearing loss in humans and mice. GATA3 haploinsufficiency causes progressive morphological degeneration starting with the outer hair cells (OHCs) at the apex and ultimately affecting all hair cells and supporting cells in the entire cochlea (Van der Wees et al., 2004; van Looij et al., 2005).

Embryonic kidney

Gata3 is expressed in the embryonic kidney. Inactivation of Gata3 by insertion of an Ires-GFP reporter gene resulted in a massive increase in nephric duct cellularity, which was accompanied by enhanced cell proliferation and aberrant elongation of the nephric duct. The nephric ducts of Gata3-/- embryos are hypercellular and fail to elongate along the normal rostrocaudal path, thereby preventing metanephros induction (Grote et al., 2006).

Epidermis and hair follicle

Expression of Gata3 appears in the developing vibrissae follicles at E14.0 and in the whole body epidermis at E15.0. Suprabasal cells of the epidermis and inner root sheet (IRS) layer of the hair follicle (HF) showed the strongest expression of Gata3 in the skin. In addition Gata3 can be found in the basal cells of the epidermis, in the infundibulum, sebaceous glands and ORS (Kaufman et al., 2003; Kurek et al., 2007).

Experiments with grafted pharmacologically rescued Gata3 -/- skin and skin specific deletion of Gata3 showed aberrations in hair follicle morphogenesis that included structural defects in the IRS and hair shaft (Kaufman et al., 2003; Kurek et al., 2007). Mice with the Gata3 deficiency in the skin have impaired hair follicle cycle and showed epidermal hyperproliferation and hyperkeratosis. Instead of making new hairs HF appears to contribute to epidermal thickening in addition to the increased proliferation of the epidermal cells (Kurek et al., 2007).

p63 is critically involved in the regulation of epidermal differentiation (King and Weinberg, 2007; Koster and Roop, 2004). p63 binds and transactivates the GATA3 promoter and in p63 null embryos GATA3 is downregulated, suggesting that in vivo p63 contributes to GATA3 transcription (Chikh et al., 2007).

Mammary glands

In the mammary epithelium of pubertal mice Gata3 was found in the luminal cells of mammary ducts and the body cells of terminal end buds (TEBs). Conditional deletion of Gata3 at different stages of development unraveled Gata3 has essential roles in the morphogenesis of the mammary gland in both the embryo and adult. Gata3 deficiency leads to an expansion of luminal progenitors and a concomitant block in differentiation. After Gata3 loss, adult mice exhibited undifferentiated luminal cell expansion with basement-membrane detachment, which led to caspase-mediated cell death in the long term. Gata3 maintains luminal epithelial differentiation in the adult mammary gland, which raises important implications for the pathogenesis of breast cancer (Asselin-Labat et al., 2007; Kouros-Mehr et al., 2006).

Human disorder - HDR

The HDR syndrome caused by GATA3 haploinsufficiency is an autosomal dominant condition, defined by the triad hypoparathyroidism, sensorineural deafness, and renal insufficiency. The hypoparathyroidism is characterized by symptomatic or asymptomatic hypocalcemia with undetectable or inappropriately normal serum concentrations of parathyroid hormone (PTH), and normal brisk increases in plasma cAMP in response to PTH infusion, which indicates normal sensitivity of the PTH receptor (Bilous et al., 1992). The sensorineural deafness is usually bilateral, although the hearing loss may vary in its severity (Bilous et al., 1992; Hasegawa et al., 1997; Muroya et al., 2001). The renal tract abnormalities, which may be uni- or bi-lateral, consist of renal cysts that may cause pelvicalyceal deformities and/or compression of the glomeruli and tubules that may lead to kidney failure; renal aplasia or hypoplasia; and vesicoureteral reflux (Bilous et al., 1992; Hasegawa et al., 1997; Muroya et al., 2001; Van Esch and Bilous, 2001; Van Esch et al., 1999; Van Esch et al., 2000).There are multiple cases of HDR where different mutations of the Gata3 gene result in the deletion of one allele of GATA3, or in a truncation of protein, or a loss of interaction with other proteins, or alter DNA-binding affinity all of which leading to GATA3 haploinsufficiency (Muroya et al., 2001; Nesbit et al., 2004; Van Esch et al., 2000).

Breast cancer

GATA3 plays an important role in hormone-dependent breast cancer. Its expression is highly correlated with the expression of the estrogen receptor alpha (ER alpha) in human breast tumors (Hoch et al., 1999). GATA3 and ER alpha regulate their own expression in hormone-dependent breast cancer cells. GATA3 is required for estradiol stimulation of cell cycle progression in breast cancer cells and directly positively regulates the expression of the ER alpha gene. Conversely the ER alpha directly stimulates the transcription of the GATA3 gene, and thus these two factors are involved in a positive cross-regulatory loop (Eeckhoute et al., 2007).

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The surface of our body is covered with skin epidermis and its appendages, which make a protective barrier that keeps microbes out and body fluids in. Mammalian skin is composed of three main layers, the epidermis, dermis and adipose layers. The epidermis forms a physical barrier protecting the organism from the environment and consists of tightly packed sheets of keratinocytes. Fundamental to the formation of epidermis and hair follicles is the ability of cells to attach to each other, thus enabling them to withstand mechanical stress.
A number of tissue specific genes are expressed in the epidermis and hair follicle. Depending on the gene disrupted and the structures affected, the consequences of gene ablation ranged from severe epidermal defects to the loss of terminal differentiation and lack of correct hair follicle formation and hair growth.

Epidermis
The epidermis is a stratified squamous epithelium in which keratinocytes are organized in distinct cell layers. During embryogenesis the single-layered embryonic ectoderm becomes a bilayered epithelium as a layer of peridermal cells is formed on its surface. Upon stratification of the epidermis, the periderm is gradually degraded (Holbrook, 1983). In adult skin the basal keratinocytes proliferate and suprabasal cells undergo terminal differentiation, viz. the formation of the cornified layers (Figure 1). The basal layer is the innermost layer of the epidermis, and contains small round cells called basal cells. The basal cells continually divide, and new cells constantly push older ones up toward the surface of the skin, where they are eventually shed. The squamous cell layer is located above the basal layer, and is also known as the stratum spinosum. The keratinocytes from the squamous layer are pushed up through the thin epidermal layer called the stratum granulosum and the stratum corneum is the outermost layer of the epidermis, and is made up of thin layers of continually shedding, dead keratinocytes.

Dermis
The dermis is located beneath the epidermis and is the thickest layer of the skin. The main functions of the dermis are to regulate temperature and to supply the epidermis with nutrient-saturated blood. This layer contains most of the skins' specialized cells and structures, including: blood and lymph vessels, hair follicles, sweat glands, sebaceous glands, nerve endings and muscles. The dermis is held together by collagen, made by fibroblasts.

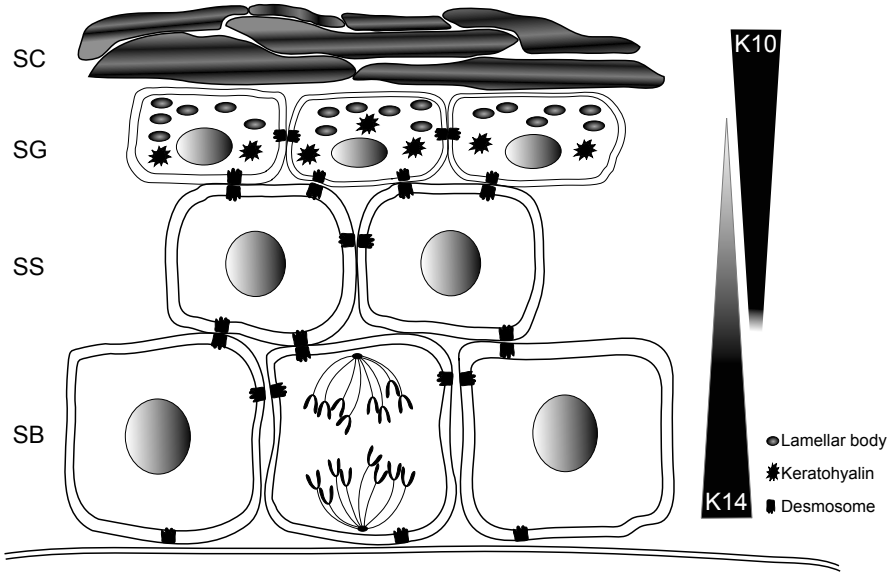

Figure 1. Epidermal differentiation. Dividing cells in the basal layer undergo terminal differentiation, move outwards and slough from the skin surface. As they move towards the skin surface they switch the expression of keratins K14 and K5 to K1 and K10. The first suprabasal cells are known as spinous cells, reflecting their cytoskeleton of K1/K10 filament bundles connected to desmosomes. As spinous cells progress to the granular layer, they produce keratohyalin granules packed with profilaggrin. Upon calcium induction in stratum granulosum, transglutaminase mediate cross-linking of the cornified envelope proteins to create a strong proteinaceous sac. The last step of terminal differentiation involves the destruction of cellular organelles and the extrusion of lipid bilayers.
Abbreviations: SB, stratum basale; SS, stratum spinosum; SG, stratum granulosum; SC, stratum corneum.

Hair follicle

The mature hair follicle (HF) in mammals has all the anatomic complexity of a miniature organ. Although most of the HF is epithelial in origin, it also contains specialized mesenchymal cells (dermal sheath and papilla), neural crest-derived melanocytes, and circulating immune cells such as Langerhans cells. Adding further to this complexity, each individual HF has its own innervation, vasculature, and musculature. The epithelial compartment of the growing HF is comprised of three concentric structures: the outer root sheath (ORS), the inner root sheath (IRS), and the central hair shaft (Figure 2). The matrix is a proliferative zone located at the proximal end of the HF surrounding the dermal papilla (DP). With the exception of the ORS, all cells are produced by the matrix.

The IRS is separated from the ORS by the companion layer. The IRS contains three different cellular layers: an externally located layer of Henle which flanks the ORS, a central layer of Huxley, and an inner layer of cuticle cells which covers the hair shaft cuticle.

The hair shaft is made up of three layers as well: the cuticle - a layer of dead,

transparent cells arranged in overlapping patterns; the cortex - thick layer of keratinized cells, longitudinally packed with keratin filaments, and the medulla, made up of large, loosely connected keratinized cells, often distinctively coloured and interspersed with air pockets. Hair-fibre thickness and shape strongly correlate with the number and size of keratinocytes in the hair medulla. The thickness of the hair medulla is controlled by the activity of the Edar, IGF, and FGF signaling pathways (Mustonen et al., 2004; Schlake, 2005; Weger and Schlake, 2005a, b; Zhang et al., 2003); however, the downstream targets and mechanisms involved in regulating hair shape and formation of hair shaft bending remain largely unknown. BMP signaling controls HF size and phenotype of the produced hairs, at least in part by regulation of the expression of cell cycle-dependent kinase inhibitor p27Kip1 in hair matrix keratinocytes.

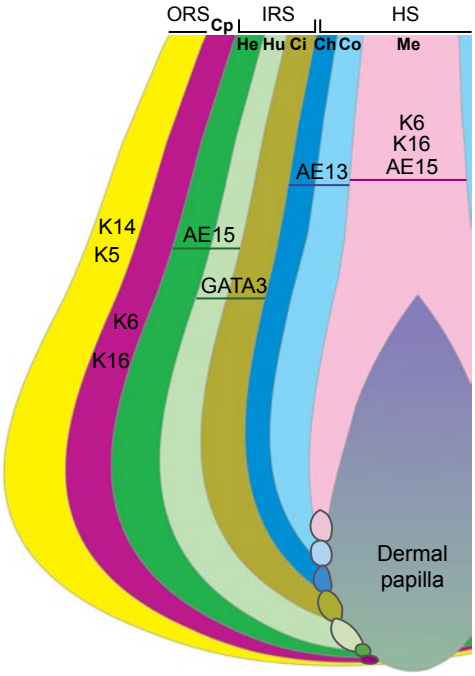

Figure 2. Schematic of the hair follicle bulb. The diagram shows the distinct cell layers of the hair follicle: ORS, outer root sheath; Cp, companion cell layer; IRS, inner root sheath; He, Henle's layer; Hu, Huxley's layer; Ci, cuticle of IRS; Ch, cuticle of hair shaft; Co, cortex of hair shaft; Me, medulla; and dermal papilla. The illustration also summarizes immunofluorescence patterns based on expression of antibodies against known markers that distinguish the complex programs of differentiation. Names of the marker proteins are indicated except for the following: AE13, Ab against hair-specific keratins that are expressed in the Co; and AE15, Ab against trichohyalin, found in all three IRS layers and the Me.

A new IRS and hair shaft develop from the hair matrix cells with each anagen cycle; (Fuchs et al., 2001; Fuchs and Raghavan, 2002; Stenn and Paus, 2001). The differentiation of the IRS cell layers is characterized by a sequence of morphological events that occur as IRS cells migrate from the hair matrix upward. These events are the production of keratin filaments, followed by the appearance of trichohyalin granules, the disintegration of the nucleus and cellular organelles, and finally the complete condensation with filaments and matrix material, which is referred to as "hardening."

The ORS is the only permanent layer within the follicle. ORS cells differentiate laterally from the basal layer that rests on a basement membrane. This is in contrast to the other cell layers of the follicle, which originate and differentiate from the bottom upward.

The development and cycling of the hair follicle is a powerful model system to study numerous developmental processes such as morphogenesis, patterning, epithelial differentiation, epithelial-mesenchymal interactions, stem cell dynamics, and homeostasis.

Development of hair follicle

Morphogenesis

After gastrulation, a single layer of neuroectoderm covers the embryo and later will give rise to the nervous system and skin epithelium. Wnt signaling, which blocks the ability of ectoderm to respond to fibroblast growth factors (FGFs), is the key factor in the development of epidermis. In the absence of FGF signaling the cells express bone morphogenic proteins (BMPs), and become fated to develop into epidermis. In the embryo, hair follicle morphogenesis begins when specialized dermal cells organize in small clusters (dermal papilla) directly beneath the epidermal layer, stimulating the keratinocytes in the epidermis to form placodes, divide and penetrate into the mesoderm, eventually giving rise to different parts of the hair follicle (Figure 3). The dermal papilla is the signaling mesodermal center for the hair follicle development (Schmidt-Ullrich and Paus, 2005). Key components regulating these mesenchymal-epidermal interactions include FGFs, BMPs and Wnt signaling. FGFs and BMPs control the placode density and Wnt signaling controls the placode formation and downgrowth. Wnt signaling also regulates de novo hair follicle regeneration after wounding and these follicle arise from epithelial cells outside of the hair follicle stem cell niche (Gat et al., 1998; Ito et al., 2007).

Hair follicle cycle

A fascinating property of hair follicles is the unique developmental cycle they undergo throughout life, with phases of active hair growth (anagen), involution (catagen) and quiescence (telogen) (Figure 3) (Hardy, 1992).

- **Anagen**

Each hair cycle starts with a growth phase – anagen. In the hair bulb matrix cells are actively producing precursor cells that move upwards and differentiate into one of the six lineages of the inner root sheath and hair shaft. The duration of anagen determines the hair length. Mice bearing naturally occurring or site directed inactivation of FGF-5 display an abnormal long hair phenotype (Hebert et al., 1994). ORS cells generate a signal for the termination of anagen.

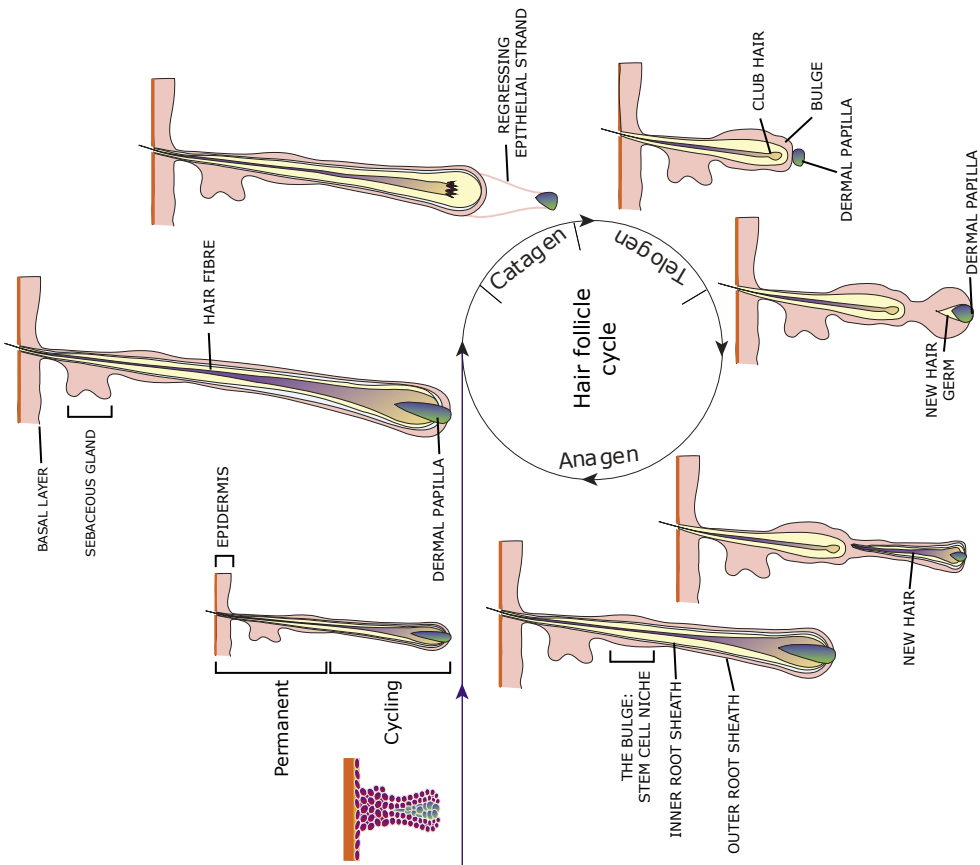

Figure 3. Hair follicle cycle and anatomy. The hair follicle morphogenesis starts when dermal papilla cells organize in clusters beneath the epidermal layer, stimulating the keratinocytes in the epidermis to form placodes, divide and penetrate into the mesoderm, giving rise to the hair follicle. The hair follicle cycle consists of stages of hair growth (anagen), follicle regression (catagen), and hair shedding (exogen) and rest (telogen). Follicles progress synchronously to the destructive (catagen) phase, during which the lower portion of the follicle undergo apoptosis and regress. As the lower follicle recedes, the epithelial strand connects the dermal papilla to the upper part of the hair follicle by dragging it upwards until the DP reaches the cells that surround the remnant club hair.
The entire lower epithelial structure is formed during anagen. The transient portion of the follicle consists of matrix cells in the bulb that generate seven different cell lineages, three in the hair shaft, and four in the inner root sheath.

The cytoskeletal protein K17 and the cytokine TNFα are regulators of anagen-catagen transition. Expression of K17 protein is required for the persistence of the anagen (growth) state in hair follicles and TNFα/TNFR1-dependent signaling is required for the timely occurrence of the anagen–catagen transition (Tong and Coulombe, 2006)

- **Catagen**

During catagen the lower portion of the follicle undergoes involution. Catagen lasts 3-4 days in mice. Apoptosis starts in early-catagen in the HF matrix cells progressing upwards in the outer and inner root sheaths to form epithelial strand which drags the dermal papilla upwards to rest below the permanent upper follicle (late catagen). During catagen a specialized structure in the HF is formed, the club hair, that connects the proximal part of the hair shaft with surrounding HF epithelium and anchors hair in the telogen HF. The club hair is formed by the ORS cells, anchoring the hair shaft to the follicle and allowing it to be retained during subsequent hair cycles. The dermal papilla is closely adjacent to the regressing HF (Botchkareva et al., 2006; Lindner et al., 1997). Hairless, which is expressed in the ORS and matrix, is a transcriptional repressor. When mutated, the epithelial strand is altered, leaving the dermal papilla behind blocking new hair growth (Potter et al., 2001).

- **Telogen**

During telogen, the hair follicle is quiescent and is comprised of a layer of ORS cells surrounding a completely formed hair. In adult mouse hair follicles, telogen can persisit for 30-40 days terminating with the initiation of a new anagen phase.
The transition from telogen to anagen occurs when stem cells at the base of the telogen follicle are activated by the signals from the dermal papilla to produce a new hair shaft. The new follicle forms adjacent to the old pocket that harbors the club hair, which will eventually be shed (exogen).

Epidermal stem cells

The epidermis is organized into epidermal proliferative units (EPUs) that self-renew for extended periods. In the interfollicular epidermis cells are generated through proliferation that occurs only in the basal layer. The slower cycling central cell and more rapidly proliferating surrounding cells constitute approximately 10 basal cells and are roughly organized into a hexagonal unit, which lies beneath a single squame (Mackenzie, 1970; Potten, 1974; Potten and Allen, 1975). EPU generates the rapidly proliferating cells, termed transient or transit amplifying (TA) cells, which move laterally and then differentiate and move upward (Kaur, 2006). Thus, within the epidermis, the main source of cells, that is the stem cells, responsible for continual epidermal renewal appear to reside in the center of the EPU (Ito et al., 2005).

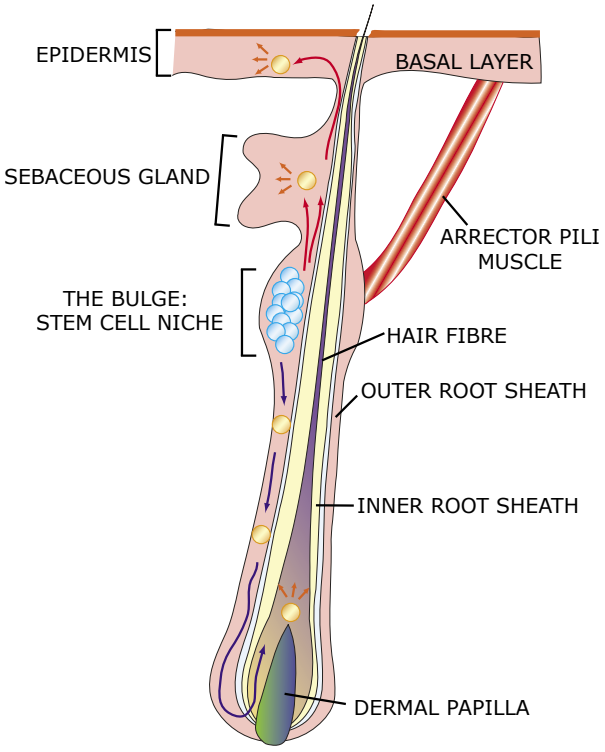

Figure 4. The follicle stem cell niche. The niche of the follicular stem cells is a restricted region of the permanent portion of the hair follicle adjacent to the attachment site of the arrector pili muscle. The bulge contains infrequently cycling, label-retaining cells, which include multipotent stem cells (blue), that are required for hair follicle cycling (blue downward arrow) and repair the epidermis on injury (red upward arrow).

Although no new hair follicles are made postnatally, the lower portion of the hair follicle regenerates in order to produce a new hair. For this purpose, and for the maintenance of the epidermis and sebaceous gland, reservoirs of multipotent epithelial stem cells are set-aside during development. Hair follicle stem cells are found in the lowest permanent portion of the hair follicle – the 'bulge' (Figure 4) (Oshima et al., 2001; Taylor et al., 2000). The bulge is a well-protected structure that remains unaffected by normal hair cycling or damage; it is the site of slowly cycling cells having a high proliferative capacity (Cotsarelis, 2006; Cotsarelis et al., 1990; Morris and Potten, 1994); and bulge cells have been shown to be multipotent (Oshima et al., 2001; Taylor et al., 2000). Follicle stem cells are activated at the telogen-to-anagen transition, to initiate a new round of hair growth (Alonso and Fuchs, 2003).

Stem cells in the hair follicle have the ability to self-renew and to generate transient-amplifying cells that can rapidly proliferate for a limited number of divisions, giving

rise to the various differentiated cells of the hair follicle. (Lavker and Sun, 2000; Morris and Potten, 1994; Potten and Bullock, 1983; Watt, 1998). Upon the signal from dermal papilla, stem cells are activated to migrate into bulb and produce a germinative layer surrounding dermal papilla. These cells now begin to proliferate rapidly, and become the transient-amplifying daughter cells that are fated to form the new hair follicle (Blanpain et al., 2004; Legue and Nicolas, 2005; Tumbar et al., 2004). Stem cells in the hair follicle bulge do not normally contribute to the epidermis which renewal is controlled by stem cells located in the basal epidermis. However, after epidermal injury cells from the bulge are recruited into the epidermis (Ito et al., 2005). Moreover, after large wounding, hair follicles form de novo in genetically normal adult mice from epithelial cells outside of the stem cells niche. Lineage analysis demonstrated that these follicles arise from epidermal cells in the wound and this process is Wnt signaling dependent (Ito et al., 2007).

Additionally, the bulge region is biochemically and molecularly distinct. One of the best (most specific) marker for mouse hair follicle bulge cells is CD34 expression as first defined by (Trempus et al., 2003). CD34, which is also a hematopoietic stem cell marker in the human, but not the mouse, highlights the bulge cells specifically within the cutaneous epithelium. Although it is also expressed by cells in the dermis, it is a cell surface protein, and antibodies recognizing CD34 were used to collect viable bulge cells by fluorescent activated cell sorting (FACS) (Blanpain et al., 2004; Trempus et al., 2003). Keratin 15 expression in human bulge cells was first described by (Lyle et al., 1998). K15 mRNA and protein are reliably expressed at high levels in the bulge, but lower levels of expression can be detected in the basal layers of the ORS and the epidermis (Lyle et al., 1998; Waseem et al., 1999). K15 expression in the epidermis is prominent in neonatal mouse and human skin but decreases with age (Liu et al., 2003; Webb et al., 2004). However, a K15 promoter used for generation of transgenic mice possesses a pattern of activity restricted to the bulge in the adult mouse (Liu et al., 2003).
Microarray profiling was used to identify differentially expressed genes in the bulge. The potential candidate hair follicle stem cells markers include β1-integrin, keratin 15, keratin 19, CD34, CD71, S100A4, S100A6, Lhx2, Tcf3, nestin and p63 (Ito and Kizawa, 2001; Jones and Watt, 1993; Li et al., 2003; Lyle et al., 1998; Michel et al., 1996; Nguyen et al., 2006; Pellegrini et al., 2001; Rhee et al., 2006; Tani et al., 2000; Trempus et al., 2003).
Some of bulge markers are common for stem cells in other tissues e.g. cell surface protein CD34 marks haematopoietic stem cell as well as endothelial progenitors, structural protein keratin 19 is used as a mammary and liver stem cell marker and Tcf3 is necessary to limit the steady-state levels of Nanog mRNA, protein, and promoter activity in self-renewing ESC (Pereira et al., 2006; Woodward et al., 2005).

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DOROTA, GATA 3 IS EXPRESSED IN THE SKIN!
NO!! ARE YOU KIDDING?!
..SKIN... SKIN... WHERE TO START? .....?..?..
I KNOW!!! I WILL DO MICRO-DISSECTION & THEN MICRO-ARRAY! IT SOUNDS REASONABLE!
HEY, KARL. DO YOU KNOW ANYTHING ABOUT LCM?
HHMMN... I'LL BE HAPPY TO SHOW YOU.
GEORGE. I HEARD THAT YOU KNOW A LOT ABOUT MICROARRAYS?
Show it to me, baby!
MAMA!!!
..GATA 3 IS IMPORTANT FOR THE SKIN..
BORREL!!!!
MY FIRST PUBLICATION!!!

Chapter 2

Transcriptome and phenotypic analysis reveals Gata3-dependent signalling pathways in murine hair follicles

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Transcriptome and phenotypic analysis reveals Gata3-dependent signalling pathways in murine hair follicles

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Running title: Gata3 in mouse epidermis and hair follicle

Key words: Gata3, Hair follicle, Epidermis, Laser capture microscopy, Microarray, Mouse

The transcription factor Gata3 is crucially involved in epidermis and hair follicle differentiation. Yet, little is known about how Gata3 co-ordinates stem cell lineage determination in skin, what pathways are involved and how Gata3 differentially regulates distinct cell populations within the hair follicle. Here, we describe a conditional Gata3$^{-/-}$ mouse (K14-Gata3$^{-/-}$) in which Gata3 is specifically deleted in epidermis and hair follicles. K14-Gata3$^{-/-}$ mice show aberrant postnatal growth and development, delayed hair growth and maintenance, abnormal hair follicle organization and irregular pigmentation. After the first hair cycle, the germinative layer surrounding the dermal papilla was not restored; instead, proliferation was pronounced in basal epidermal cells. Transcriptome analysis of laser-dissected K14-Gata3$^{-/-}$ hair follicles revealed mitosis, epithelial differentiation and the Notch, Wnt and BMP signaling pathways to be significantly overrepresented. Elucidation of these pathways at the RNA and protein levels and physiologic endpoints suggests that Gata3 integrates diverse signaling networks to regulate the balance between hair follicle and epidermal cell fates.

Introduction

The hair follicle (HF) comprises a discrete anatomical and physiological entity composed of epidermal stem cells that give rise to proteinaceous fibers (i.e. hair shafts) in response to growth factors (Schmidt-Ullrich and Paus, 2005). The HF is a mammalian organ that undergoes cyclic transformations, thereby allowing the study of fundamental developmental processes (e.g. proliferation, differentiation). The hair shaft and adjacent multi-layered inner root sheath (IRS) originate from the epithelial matrix that surrounds the dermal papilla, the permanent mesenchymal portion of the HF. In response to signals from the papilla, proliferating epithelial cells protrude into the dermis forming the first HF. Undifferentiated matrix cells remain

in the hair bulb interacting with the dermal papilla (Cotsarelis et al., 1990; Oliver and Jahoda, 1988), or else differentiate as they migrate towards the epidermis.

An outer root sheath (ORS) surrounds the follicle that is contiguous with and biochemically similar to the basal epidermal layer. The bulge region is located beneath the sebaceous gland of the HF, marking the lowest point of the upper part of the follicle that is permanent. It contains a reservoir of stem cells able to repopulate HF lineages (Fuchs et al., 2004; Morris et al., 2004; Oshima et al., 2001; Taylor et al., 2000). HFs undergo self-renewal throughout life. Each hair cycle starts with a growth phase (anagen) where a germinative layer is formed around the dermal papilla derived from stem cells present in the bulge (Morris et al., 2004). Then, during catagen, the lower epithelial part regresses followed by resting and shedding periods (the telogen and exogen phases, respectively). Eventually, the lower part of the follicle grows downwards again to generate a new hair (Hardy, 1992).

Gata3, a zinc finger transcription factor essential for the proper development of various tissues and organs (Pandolfi et al., 1995) is known to be involved in HF development and skin cell lineage determination (Ellis et al., 2001; Kaufman et al., 2003). Using skin transplantation experiments, Gata3 was shown to be critically involved in skin cell lineage determination whereas its absence resulted in dysfunctional IRS precursor cells that could not differentiate properly (Kaufman et al., 2003). To examine how GATA3 exerts its role in IRS cell lineage determination and to identify potential Gata3 target genes, we generated K14-Gata3$^{-/-}$ mice to specifically ablate Gata3 expression, thereby revealing its central role in mouse epidermis and HF development.

Material and methods

Targeting and homologous recombination

The Gata3 conditional knockout construct is shown in Fig. 1. E14 ES cells were electroporated, screened and germ line chimaeric mice generated by blastocyst injection. The PGKPuro cassette was removed by flp recombinase flpe6 (Buchholz et al., 1998)

Laser-capture microdissection and microarray analysis

Laser-capture microdissection was performed on 10 µm cryosections using the PALM MicroBeam microscope system (PALM Microlaser Technologies). RNA was analysed as described (http://www.affymetrix.com/index.affx). Microarray chips were analyzed with Affymetrix GeneChip software. Microarray data complied with the MIAME regulations and are available in ArrayExpress (accession code: 5988).

In situ hybridization, TUNEL and skin barrier

In situ hybridization with antisense digoxigenin-UTP-labelled RNA probes on 10 µm sections of skin samples was performed as described (Schaeren-Wiemers and Gerfin-Moser, 1993). Cell death was detected with the Cell Death Detection Kit, Fluorescein (Roche). Skin barrier experiments were performed as described (Hardman et al., 1998).

Immunohistochemistry, X-gal staining and BrdU labelling
Mice were injected with 50 mg/kg bodyweight BrdU and sacrificed 2 hours later. Cryosections were fixed for 10 minutes in 4% PFA in PBS, then blocked with 1% BSA, 0.05% Tween in PBS. Primary antibodies were: K6 (rabbit, 1:5,000; Covance, PRB-169P), K14 (rabbit, 1:10,000; Covance, PRB-155P), Gata3 (mouse, 1:200; Santa Cruz, HCG3-31), CD3 (rat, 1:100; Santa Cruz, KT3), cyclin A2 (rabbit, 1:100; Santa Cruz, C-19), Cdk4 (rabbit, 1:100; Santa Cruz, C-22), cyclin E1 (rabbit, 1:100; Santa Cruz, M-20), loricrin (rabbit, 1:500; Covance, PRB 145P), K14 (rabbit, 1:10,000: Covance, PRB_155P), ß-catenin (mouse, 1:100; BD, #14), AE13 (mouse, 1:20) (Lynch et al., 1986), AE15 (mouse, 1:10) (O'Guin et al., 1992), MTS24 (rat, 1:200) (Gill et al., 2002), BrdU (mouse, 1:100; DAKO, #Bu20a) and K10 (mouse, 1:50; Sigma, #k8.60). Relevant FITC-, TxR- or HRP-conjugated goat secondary antibodies (1:100, DAKO) were used. For BrdU immunohistochemistry, tissue was fixed in 4% PFA, 4°C overnight and subsequently embedded in paraffin and sectioned at 7 µm. After deparaffination, sections were boiled in 0.01 M citrate buffer (pH 6.0) for 15 minutes prior to primary antibody incubation. For X-Gal staining, sections were fixed for 1 minute in 0.5% glutaraldehyde, 1% PFA, and incubated in X-Gal staining solution for 5 hours at room temperature. Images were taken with an Olympus BX40 microscope and Axio Imager (Zeiss) fluorescence microscope.

Results

Generation of keratinocyte-specific Gata3$^{-/-}$ mice (K14- Gata3$^{-/-}$)

The role of Gata3 in HF development was examined in mice expressing human keratin 14 promoter-driven Cre recombinase that restricts expression in the basal layer of stratified squamous epithelia and the ORS of HFs (Jonkers et al., 2001; Vassar et al., 1991). These mice were crossed with conditional Gata3 knockout (Gata3cko) mice (Fig. 1A). The human K14 promoter drives Cre expression (visualized in a floxed Rosa-LacZ reporter mouse line) in epidermis, all HF cell types, salivary gland, mammary epithelium and epithelial cells from other tissues (Jonkers et al., 2001). We detected Cre activity in the epidermis at embryonic day (E) 13.5, whereas placodes were Cre-positive two days before the onset of Gata3 expression (i.e. E15.5). Heterozygous skin-specific Gata3 knockouts were indistinguishable from wild-type (wt) littermates (not shown). K14cre/Gata3cko/wt mice were crossed with heterozygous and homozygous Gata3cko mice to generate K14cre/Gata3cko mice (referred to as K14-Gata3$^{-/-}$ or ko mice). These lacked expression of Gata3 in the hair follicles and epidermis at all stages, as determined by immunohistochemistry (not shown).

Aberrant postnatal growth and delayed development in K14-Gata3$^{-/-}$ mice.

At birth, K14-Gata3$^{-/-}$ mice were morphologically and histologically indistinguishable from wt littermates, ruling out severe impaired embryonic development. However, from postnatal day (P) 2 onwards, K14-Gata3$^{-/-}$ mice demonstrated aberrant growth and delayed development resulting in dwarfism (Fig. 1B, C) and weight loss

(Fig. 1D) during the first month of life. Further analysis revealed a half-opened-eye phenotype (Fig. 1E, F), stiff hind legs (Fig. 1G) and absence of visible nipples in K14-Gata3$^{-/-}$ mice (Fig. 1H, I). Importantly, K14-Gata3$^{-/-}$ hair development was delayed, hair growth was abnormal and replacement was sparse, if not absent, gradually resulting in bald mice (Fig. 1G).

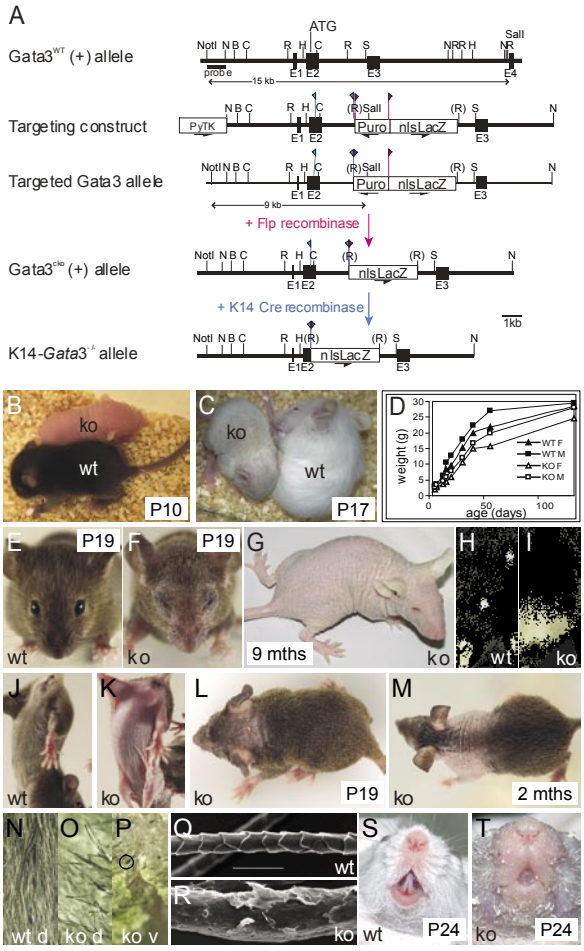


Figure 1. (A) Generation of floxed/deleted Gata3 alleles. LoxP sites were introduced in the start codon (in exon 2) and in the second intron (blue flags) of Gata3. A Puro cassette, flanked by FRT sites (pink flags) and coupled to an nlsLacZ gene lacking a polyA signal was introduced in intron 2. Arrows indicate lengths of NotI-SalI restriction enzyme fragments that hybridize to the external 5' probe. A Polyoma thymidine kinase gene (PyTK) was added 5' to the construct to counter select against random integration. Flp recombinase mice (Buchholz et al., 1998) were used to remove the Puro cassette. After recombination by Cre recombinase, resulting in excision of the part of exon 2 that is 3' to the start ATG of Gata3 and the part of intron 2 that is 5' to the EcoRI site, the nlsLacZ reporter replaces the 3' end of the truncated Gata3 gene. Arrows below genes indicate direction of transcription. Black boxes correspond to exons. B, BamHI; C, ClaI; H, HindIII; N, NcoI; R, EcoRI; S, SacI. (B-T). Phenotypes caused by homozygous deletion of Gata3 in the skin (ko) versus wt. (B) Delayed hair growth in K14-Gata3$^{-/-}$ at P10. (C) Short stubby hairs instead of normal coat of fur. (D) Weight difference in the first months of life. (E,F) Eyes are still closed, whiskers are present but rudimentary, most facial hairs have disappeared and remaining hairs are still very short. (G) After 3 months, most K14-Gata3$^{-/-}$ mice are completely bald; eyes are still closed. (H,I) The absence of visible nipples in female K14-Gata3$^{-/-}$ mice. (J,K) The complete loss of hairs from the abdominal area at 2 months. (L,M) Baldness starts at the head and progress from anterior to posterior. (N-P) Coat of fur at P26. d, dorsal; v, ventral. (N) wt back hair; (O) K14-Gata3$^{-/-}$ back hair; (P) K14-Gata3$^{-/-}$ belly hair (and flakes; note the encircled, thick rounded tip of the hair instead of the normal fine hair tips). (Q,R) Scanning electron microscopy of dorsal wt (Q) and K14-Gata3$^{-/-}$ (R) hairs of 12-week-old mice. Note thickness of K14-Gata3$^{-/-}$ hair as well as absence of regular cuticle cells and presence of irregular flakes. (S,T) Around P24, K14-Gata3$^{-/-}$ skin starts flaking. Scale bar in Q: 50 µm for Q,R.

Delayed hair growth and disturbed hair maintenance in K14-Gata3$^{-/-}$ mice

In contrast to wt, where external hairs were visible from P7, forming a well-developed fur coat by about P10, K14-Gata3$^{-/-}$ mice remained completely bald (Fig. 1B). From P12 onwards, hair growth was sparse around the head and neck resulting in a thin fur coat with short and stubby hairs (P17, Fig. 1C). The delayed onset of hair growth was followed by the gradual abdominal hair loss (P19, Fig. 1J, K) and an anterior to posterior hair shedding in the head area (Fig. 1L, M) with limited hair regeneration. Remaining hairs appeared irregular with a short, thick and hard appearance and a thick-rounded tip instead of the normally observed thin tip (Fig. 1N-R). Whiskers appeared short and thick (Fig. 1E, F, S, T).

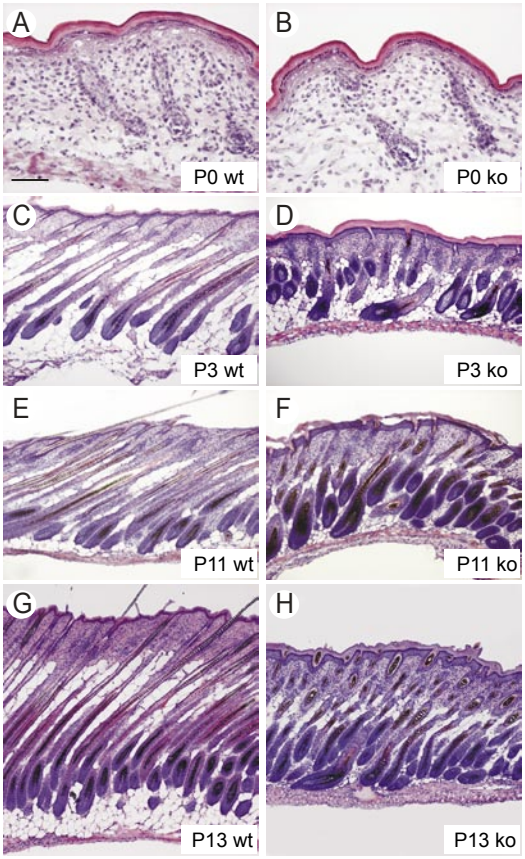


Figure 2. Haematoxylin-Eosin staining of fresh-frozen dorsal skin sections of control and K14-Gata3$^{-/-}$ mice. (A,B) P0: skin of K14-Gata3$^{-/-}$ is indiscernible from control skin. (C,D) P7: in K14-Gata3$^{-/-}$ HFs are disorganized and do not produce hairs. Epidermis is thickened. (E,F) P11: in the mutant, (abnormal) hairs are still not penetrating the epidermis. (G,H) P13: some irregularly formed hairs penetrate the skin; both dermis and subcutaneous fat layer (white cells) are much thinner in K14-Gata3$^{-/-}$ than in control skin. Scale bar in A: 50 μm for A,B; 200 μm for C-H.

Aberrant hair follicle organization and abnormal hair pigmentation in K14-Gata3$^{-/-}$ mice.

At P0, K14-Gata3$^{-/-}$ HFs appeared normal. However, beginning at P3, K14-Gata3$^{-/-}$ HFs were abnormal and grew at a much wider angle, often parallel to the skin surface, never reaching the epidermis (Fig. 2H). However, the overall number of HFs was not reduced, suggesting that Gata3 is not essential during HF induction.

Whereas wt hairs had the typical ladder-like appearance resulting from the large, keratinized pigment-containing cells interspersed with air pockets in the hair medulla, the K14-Gata3$^{-/-}$ hair pigment deposition was disorganized and without air spaces (Fig. 2F, 1R) and the sebaceous glands were enlarged from P7 onwards. Subsequently, the K14-Gata3$^{-/-}$ HF entered telogen after a delayed catagen lasting at least 6 days instead of the normal 3 days (Supplementary Fig. S1A-F) and the HF proximal end failed to reform (Supplementary Fig. S1I, J). New visible hairs were not produced, although some developing HFs and small cysts were visible (Supplementary Fig. S1J).

The newly formed HFs that derive from stem cells (Morris et al., 2004) are known to migrate from the bulge to the matrix area of the hair bulb, thereby forming a germinative layer that becomes committed to distinct HF cell lineages (Legue and Nicolas, 2005). The lack of HF renewal prompted us to investigate whether GATA3 is also expressed in cells other than those of the IRS (Kaufman et

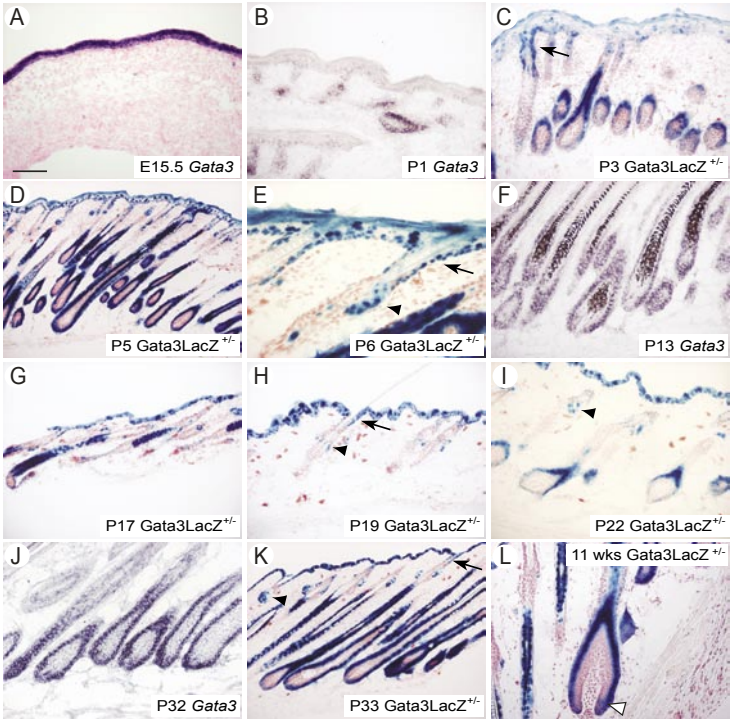


Figure 3. Expression pattern of Gata3 during the cell cycle. Gata3 expression is visualized by in situ hybridization in the control (A,B,F,J), or by X-Gal staining in the Gata3 LacZ knockin skin (C-E,G-I,K,L). At E15.5 there is strong expression of Gata3 in the epidermis (A). During anagen (A, P3), Gata3 is highly expressed in the IRS, ORS, sebaceous glands (black arrowhead), epidermis and infundibulum (arrows) (B-F,I-L,), and closer inspection (L) reveals that some cells in the germinative layer around the dermal papilla are also Gata3 positive (white arrowhead). During catagen, Gata3 expression was restricted to the IRS (G) and was absent in HF during telogen but present in the epidermis and sebaceous gland (H). Scale bar in A: 200 µm for D,K; 150 µm for A-C,F,G-J,L; 50 µm for E.

al., 2003). Evaluation of Gata3 expression in a Gata3 LacZ knockin mice and by in situ hybridization revealed GATA3 to be readily expressed in other skin structures (Pata et al., 1999, Fig. 3 and Supplementary Fig. S2). At E15.5, Gata3 was expressed in the epidermis (Fig. 3A) whereas, during anagen, Gata3 was also expressed in the IRS, ORS, sebaceous glands, epidermis and infundibulum (Fig. 3B-L, and Supplementary Fig. S2) and in certain germinative layer cells surrounding the dermal papilla (Fig. 3F, J and L). During catagen, Gata3 expression was restricted to the IRS (Fig. 3G) whereas during telogen, it was present in the epidermis and sebaceous glands, but absent from the HF (Fig. 3H).

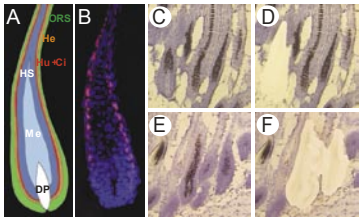
Epidermal hyperplasia and hyperkeratosis in K14-Gata3-/- skin

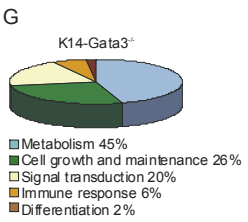
Instead of developing new HF bulbs, we noticed pronounced hyperplasia in the K14-Gata3-/- epidermis (see below). Despite a slightly delayed barrier function in fetuses at E16.5, E17.5 and E18.5 (data not shown), initial development of the epidermis appeared normal in skin sections from newborn mice (P0, Fig. 2A-B and P3, Fig. 2C-D). At P3, skin hyperplasia was followed by wrinkles (Fig. 2E-H) covered with squames (Fig. 1T). K14-Gata3-/- skin sections, at different ages, revealed gradual, pronounced epidermal thickening including that of basal and suprabasal layers (see Supplementary Fig. S3B,D) as well as the development of hyperkeratosis (increased thickness of stratum corneum, Fig. 2C-H, Supplementary Fig. S3B-C). We did not observe any nuclei in the squames of stratum corneum that would mark the presence of immaturely shed keratinocytes, a feature that is routinely observed in psoriasis. In contrast to the thickened epidermis, the dermis of K14-Gata3-/- pups appeared substantially thinner, along with a reduction in subcutaneous adipose tissue as compared with wt littermates (Fig. 2G, H).

Transcriptome analysis in K14-Gata3-/- HF

To get an unbiased insight into the HF phenotype of K14-Gata3-/- mice, we implemented a laser-capture approach (Fig. 4A-F) to isolate the proximal end of HFs from 11-day old wt and K14-Gata3-/- mouse skin (by then, the K14-Gata3-/- skin phenotype is fully developed, n=6, Fig. 4G, H) and examined their transcriptome profiles. Two-tail, pair-wise analysis of variance revealed 1516 genes with significantly different expression patterns between the two genotypes ($P<0.01$, >1.2-fold up- or downregulated (see Fig. 4H and Supplementary table S1). This substantially exceeds the ~80 genes expected to occur by chance under these selection criteria.

To avoid any preselection of genes and thus the potential introduction of bias, we used a previously described methodology (Hosack et al., 2003) to identify all over-represented biological processes in our dataset [i.e. cell cycle, epithelial growth and differentiation, immune and defense responses, signal transduction pathways and energy metabolism (Fig. 4G)). Analysis of these processes (see Fig. 4H and Supplementary table S1) led us to identify:





H

Code	Gene description	Symbol	FC	P
Cell cycle				
1417911_at	cyclin A2	Ccna2	-1.94	0.0000
1448205_at	cyclin B1	Ccnb1	-1.87	0.0000
1422513_at	cyclin F	Ccnf	-2.32	0.0005
1420827_a_at	cyclin G1	Ccng1	-1.38	0.0016
1416488_at	cyclin G2	Ccng2	-1.53	0.0034
1417131_at	cell division cycle 25 homolog A	Cdc25a	-1.60	0.0105
1421963_a_at	cell division cycle 25 homolog B	Cdc25b	-1.33	0.0042
1456077_x_at	cell division cycle 25 homolog C	Cdc25c	-1.69	0.0051
1448314_at	cell division cycle 2 homolog A	Cdc2a	-1.63	0.0002
1422016_a_at	centromere autoantigen H	Cenph	-1.58	0.0073
1439436_x_at	inner centromere protein	Incenp	-1.71	0.0009
1417299_at	NIMA -related expressed kinase 2	Nek2	-2.63	0.0000
1419620_at	pituitary tumor-transforming 1	Pttg1	-2.32	0.0009
1416161_at	RAD21 homolog (S. pombe)	Rad21	-1.42	0.0039
1417830_at	struct.maint.of chromosomes 1-l1	Smc1l1	-1.29	0.0003
1458479_at	struct.maint. of chromosomes 2-l1	Smc2l1	-1.89	0.0109
1427275_at	struct.maint. of chromosomes 4-l1	Smc4l1	-1.80	0.0037
1452954_at	ubiquitin-conjugating enzyme E2C	Ube2c	-1.85	0.0000
1424046_at	budding uninh/ed by benz/oles 1	Bub1	-1.68	0.0015
1447363_s_at	budding uninh/ed by benz/oles 1b	Bub1b	-1.90	0.0000
Epidermal differentiation				
1421589_at	keratin complex 1, acidic, gene 1	Krt1-1	3.41	0.0006
1460347_at	keratin complex 1, acidic, gene 14	Krt1-14	1.76	0.0036
1422667_at	keratin complex 1, acidic, gene 15	Krt1-15	2.91	0.0000
1448932_at	keratin complex 1, acidic, gene 16	Krt1-16	11.28	0.0000
1423227_at	keratin complex 1, acidic, gene 17	Krt1-17	2.27	0.0001
1417156_at	keratin complex 1, acidic, gene 19	Krt1-19	1.65	0.0034
1420409_at	keratin complex 1, acidic, gene 24	Krt1-24	1.40	0.0007
1427179_at	keratin complex 1, acidic, gene 3	Krt1-3	6.15	0.0002
1418742_at	keratin complex 1, acidic, gene 4	Krt1-4	9.30	0.0007
1427751_a_at	keratin complex 1, acidic, gene 5	Krt1-5	6.25	0.0000
1427365_at	keratin complex 2, basic, gene 10	Krt2-10	7.50	0.0000
1460185_at	keratin complex 2, basic, gene 18	Krt2-18	2.29	0.0001
1427290_at	keratin complex 2, basic, gene 19	Krt2-19	6.39	0.0003
1424096_at	keratin complex 2, basic, gene 5	Krt2-5	1.21	0.0022
1422784_at	keratin complex 2, basic, gene 6a	Krt2-6a	4.36	0.0000
1450536_s_at	keratin associated protein 12-1	Krtap12-1	8.70	0.0000
1419707_at	keratin associated protein 14	Krtap14	4.21	0.0015
1419507_at	keratin associated protein 15	Krtap15	5.10	0.0004
1425655_at	keratin associated protein 16-1	Krtap16-1	25.58	0.0000
1427549_s_at	keratin associated protein 16-10	Krtap16-10	6.40	0.0000
1427801_at	keratin associated protein 16-2	Krtap16-2	26.15	0.0000
1425431_at	keratin associated protein 16-3	Krtap16-3	10.22	0.0004
1426203_at	keratin associated protein 16-4	Krtap16-4	30.75	0.0000
1425430_at	keratin associated protein 16-5	Krtap16-5	32.73	0.0004
1425237_at	keratin associated protein 16-8	Krtap16-8	8.41	0.0000
1427366_at	keratin associated protein 3-1	Krtap3-1	8.09	0.0007
1452957_at	keratin associated protein 3-3	Krtap3-3	8.72	0.0005
1451859_at	keratin associated protein 6-1	Krtap6-1	8.31	0.0000
1449919_at	keratin associated protein 6-2	Krtap6-2	19.43	0.0000
1427842_at	keratin associated protein 6-3	Krtap6-3	5.22	0.0005
1427211_at	keratin associated protein 8-1	Krtap8-1	2.17	0.0006
1421689_at	keratin associated protein 8-2	Krtap8-2	14.74	0.0000
1441956_s_at	cut-like 1 (Drosophila)	Cutl1	-4.25	0.0012
Nucleosome assembly				
1455712_at	histone 3, H2bb	Hist3h2bb	5.84	0.0000
1416101_a_at	histone 1, H1c	Hist1h1c	5.06	0.0000
1430357_at	H3 histone, family 3B	H3f3b	4.32	0.0001
1425398_at	histone 1, H2bc	Hist1h2bc	4.21	0.0000
1449482_at	histone 3, H2ba	Hist3h2ba	3.10	0.0017
1435865_at	histone 3, H2a	Hist3h2a	2.89	0.0000
1418367_x_at	histone 2, H2aa1	Hist2h2aa1	2.86	0.0008
1442051_at	histone 2, H3c1	Hist2h3c1	2.61	0.0000
1455725_a_at	H3 histone 3B	H3f3b	1.24	0.0001

Code	Gene description	Symbol	FC	P
Immune and defense responses				
1455082_at	Casitas B-lineage lymphoma b	Cblb	-1.66	0.0022
1448569_at	CD8 antigen, beta chain	Cd8b	-1.51	0.0002
1418392_a_at	guanylate nucleotide binding protein 3	Gbp3	-3.42	0.0036
1449875_a_at	histocompatibility 2, T region locus 10	H2-T10	-2.51	0.0000
1449556_at	histocompatibility 2, T region locus 23	H2-T23	-4.37	0.0009
1452231_x_at	interf. activated gene 205	Ifi205	-2.89	0.0092
1450783_at	interf.-induced protein 1	Ifit1	-2.33	0.0012
1449025_at	interf.-induced protein 3	Ifit3	-5.13	0.0000
1448436_a_at	interf. regulatory factor 1	Irf1	-1.86	0.0057
1421322_a_at	interf. depend.(+) acting tr.factor 3g	Isgf3g	-2.87	0.0000
1424775_at	2'-5' oligoadenylate synthetase 1G	Oas1g	-15.19	0.0000
1453196_a_at	2'-5' oligoadenylate synthetase-like 2	Oasl2	-2.64	0.0002
1418131_at	SAM domain and HD domain, 1	Samhd1	-1.34	0.0023
1419307_at	tumor necrosis factor rec. 13c	Tnfrsf13c	-2.42	0.0001
Wnt signalling pathway				
1435373_at	casein kinase 1, epsilon	Csnk1e	1.73	0.0000
1417419_at	cyclin D1	Ccnd1	1.66	0.0006
1416123_at	cyclin D2	Ccnd2	-2.27	0.0003
1422985_at	frizzled homolog 1 (Drosophila)	Fzd1	2.94	0.0000
1450135_at	frizzled homolog 3 (Drosophila)	Fzd3	2.84	0.0000
1448662_at	frizzled homolog 6 (Drosophila)	Fzd6	1.69	0.0019
1416658_at	frizzled-related protein	Frzb	-1.54	0.0072
1449058_at	GLI-Kruppel family member GLI	Gli	-1.57	0.0005
1455277_at	Hedgehog-interacting protein	Hhip	-1.66	0.0002
1431947_at	low density lipoprotein receptor	Ldlr	-1.79	0.0017
1418634_at	Notch gene homolog 1 (Drosophila)	Notch1	1.71	0.0000
1417985_at	Notch-regulated ankyrin repeat protein	Nrarp	-2.40	0.0001
1422655_at	patched homolog 2	Ptch2	-2.84	0.0091
1418085_at	protein kinase C, zeta	Prkcz	2.12	0.0008
1451358_a_at	Rac GTPase-activating protein 1	Racgap1	-1.60	0.0000
1436869_at	sonic hedgehog	Shh	-2.37	0.0000
1426091_a_at	wingless-related MMTV integr.site 10b	Wnt10b	1.90	0.0024
1450772_at	wingless-related MMTV integr. site 11	Wnt11	3.58	0.0000
1450763_x_at	wingless-related MMTV integr. site 3	Wnt3	1.94	0.0006
1437673_at	wingless-related MMTV integr. site 5A	Wnt5a	-4.43	0.0001
1419708_at	wingless-related MMTV integr. site 6	Wnt6	-1.51	0.0053
1420006_at	bone morphogenetic protein 15	Bmp15	1.71	0.0025
1450759_at	bone morphogenetic protein 6	Bmp6	2.95	0.0000
1449873_at	bone morphogenetic protein 8a	Bmp8a	1.94	0.0002
Pro-apoptotic				
1425918_at	EGL nine homolog 3 (C. elegans)	Egln3	-3.55	0.0015
1422470_at	BCL2/adenovirus E1B inter. protein 1	Bnip3	-2.01	0.0047
1418901_at	CCAAT/enhancer binding protein beta	Cebpb	-1.80	0.0035
1437182_at	death inducer-obliterator 1	Dido1	-1.74	0.0050
1448449_at	receptor-interacting ser.-thr.kinase 3	Ripk3	-1.59	0.0080
1437503_a_at	scotin gene	Scotin-pendin	-1.49	0.0002
1451233_at	Tnf receptor-associated factor 2	Traf2	-1.47	0.0030
1449839_at	caspase 3	Casp3	-1.30	0.0088
1433699_at	TNF, alpha-induced protein 3	Tnfaip3	-1.25	0.0030
1435834_at	growth arrest specific 2	Gas2	-1.24	0.0048
1448809_at	chromosome segregation 1-like	Cse1l	-1.22	0.0079
Anti-apoptosis				
1434229_a_at	polymerase (DNA directed), beta	Polb	1.71	0.0001
1427127_x_at	heat shock protein 1B	Hspa1b	1.56	0.0008
1425281_a_at	glucocorticoid-induced leucine zipper	Gilz	1.78	0.0002
1454860_x_at	defender against cell death 1	Dad1	1.49	0.0084
1422452_at	Bcl2-associated athanogene 3	Bag3	1.89	0.0007

Figure 4. Transcriptome analysis in K14-Gata3$^{-/-}$ HF. (A-F) Schematic of HF (A) and Gata3 expression in innermost layers of IRS at P11 (B). Fresh-frozen skin sections of P11 wt (C,D) and K14-Gata3$^{-/-}$ (E,F) before (C,E) and after (D,F) collecting HF tissue by laser-capture. RNA from the captured tissue was used for microarray analysis. DP, dermal papilla; He, Henle's layer of IRS; HS, hair shaft; Hu+Ci, Huxley's layer and cuticle of IRS; Me, medulla; ORS, outer root sheath. (G,H) Full mouse genome transcriptome analysis of laser-captured, K14-Gata3$^{-/-}$ and wt HFs. (G) Pie chart of significantly over-represented biological processes in K14-Gata3$^{-/-}$ as compared with wt HFs. (H) Significant gene expression changes in the K14-Gata3$^{-/-}$ as compared with wt mouse HFs (for an extensive overview, see Table S1 in the supplementary material). FC, fold change; P=P-value for two-tail analysis of variance.

1. The downregulation of genes associated with the mitotic cell cycle and replication (Fig. 4H and Supplementary table S1) and the upregulation of genes associated with the nucleosomal assembly (Fig. 4H) suggesting that cells in the K14-Gata3$^{-/-}$ HF require GATA3 to progress successfully through the cell cycle.
2. The down- and upregulation of pro- and anti-apoptotic genes respectively (Fig. 4H

and Supplementary table S1) supporting the notion that proliferation, differentiation and apoptosis are co-regulated processes all requiring GATA3 constitutive levels in the developing HF; the dampening of apoptosis paralleled the inability of K14-Gata3$^{-/-}$ HF cells to progress through the cell cycle.
3. The upregulation of genes associated with epithelial growth and differentiation (e.g. several keratin and keratin-associated genes, Fig. 4H and Supplementary table S1) likely reflecting the presence of incompletely differentiated cell types.
4. The suppression of all genes associated with immune, inflammatory and defense responses (Fig. 4H and Supplementary table S1) and the downregulation of genes associated with fatty acid synthesis and storage (Supplementary Fig. S1) confirming the considerable loss of fat in the dermis of K14-Gata3$^{-/-}$ mice (Fig. 2H).
5. The regulation of genes associated with the FGF, Notch, WNT and BMP signaling pathways (Fig. 4H and Supplementary table S1).

PCR evaluation of the expression levels of several genes in the HF of K14-Gata3$^{-/-}$ mice and wt littermates confirmed the validity of microarray data (Supplementary Fig. S7).

Aberrant proliferation and cell cycle regulation in the K14-Gata3$^{-/-}$ hair follicle.

The suppression of genes associated with the cell cycle machinery prompted us to evaluate the proliferative capacity and apoptosis in HF. We found a significantly higher frequency of BrdU (+) cells in the K14-Gata3$^{-/-}$ basal epidermal layer at P7 (Fig. 5A-C) and P11-15 (data not shown). Subsequent analysis revealed a substantially higher frequency of BrdU (+) cells in the ORS that was continuous with the epidermis (Fig. 5D-F area "b"). In contrast to the wt HF, those cells located close to the hair bulb (i.e. hair matrix cells) most responsible for the nourishment of the growing hair (Fig. 5D-F area "a") demonstrated decreased BrdU incorporation. TUNEL (Fig. 5G-I) and caspase 3 (not shown) staining revealed reduced apoptosis in the K14-Gata3$^{-/-}$ skin, thereby confirming the previously observed suppression of pro-apoptotic genes and suggesting that aberrant cell proliferation most likely contributes to diminished hair growth. Furthermore, K14-Gata3$^{-/-}$ skin demonstrated an as yet unexplained reduction in T-cells (CD3) (Fig. 5J-L) and MHC class II cells (not shown) supporting the notion that the epidermal abnormalities are a direct consequence of Gata3 loss in the mouse skin and not secondary to inflammation (Fig. 5G-H).

The proliferative capacity of those cells located close to the hair bulb (Fig. 5E, area "a") as opposed to those located in the epithelial lining (area "b") in the K14-Gata3$^{-/-}$ HFs is in agreement with the aberrant regulation of genes associated with the cell cycle (Fig. 4H). In situ mRNA or protein expression analysis further confirmed the diminished cyclin D2, cyclin E1, cyclin A2 and cdk4 in most cells located within the hair bulb as opposed to those lying along the HF ORS as well as an increased cyclin D1 expression in the ORS (Supplementary Fig. S4).

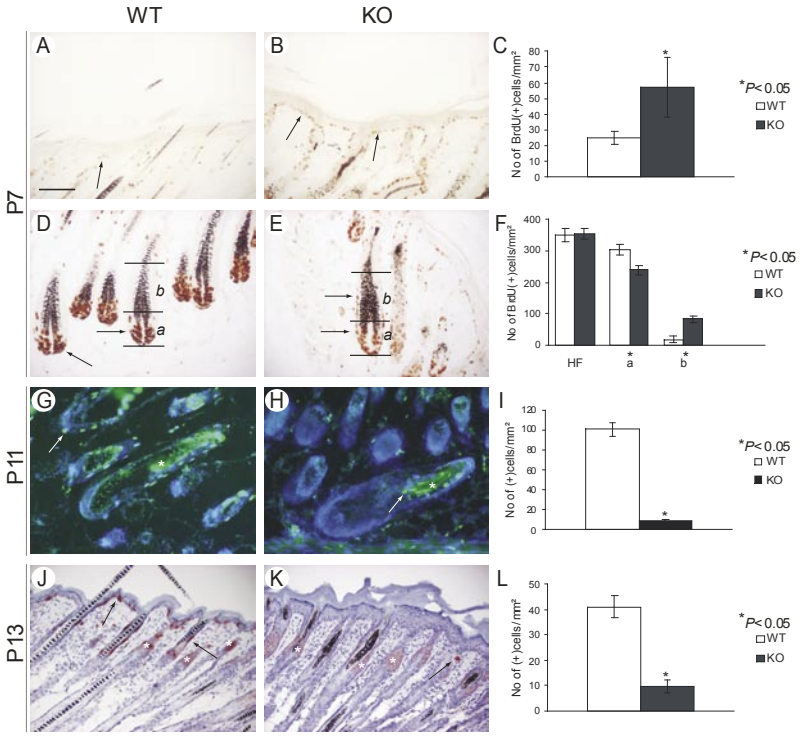


Figure 5. Altered distribution of dividing cells, T-cell number and apoptosis in K14-Gata3$^{-/-}$ skin. (A-F) BrdU labeling of dividing cells of wt (left) and K14-Gata3$^{-/-}$ (right) skin. In wt animals, BrdU labeling (brown-stained nuclei) is mostly present in the bulb of the HFs (D). The cells of the basal layer of the K14-Gata3$^{-/-}$ epidermis are dividing at a much higher rate (compare A with B). There is no obvious difference in the number of BrdU-labelled HF cells, but in their distribution (compare regions a and b in D-F). (G-I) TUNEL assay shows altered apoptosis in K14-Gata3$^{-/-}$ skin as compared with control skin at P11. Arrows indicate positive cells (J-L): K14-Gata3$^{-/-}$ skin contains less CD3 positive T-cells (red dots, arrows) than wt skin. White asterisks mark background staining of an artifact of the knots of highly keratinized material (G,H) and sebaceous glands (J,K). Data are given as mean±s. e.m.; *, P≤0.05; Student's t-test. Scale bar in A: 150 μm for A,B,D,E,J,K; 100 μm for G,H.

Keratins and keratin-associated proteins in K14-Gata3$^{-/-}$ mouse skin.

Of the 34 keratin and keratin-associated proteins whose expression was significantly altered, 30 were overexpressed in K14-Gata3$^{-/-}$ mice. In particular, we noticed the increased expression of the Krtap-16 gene family ranging from 6- to ~33-fold that was further confirmed by in situ mRNA evaluation of Krtap16-7 (Fig. 6A and B). Its expression domain was not separate from the pigmented cells, suggesting that the cortex and medulla are no longer separated (Fig. 6A-B). Consistent with previous findings, AE13 antibody detected the acidic hair keratins expressed in the cortex and cuticle of the hair shaft (Lynch et al., 1986 and Fig. 6C), whereas K14-Gata3$^{-/-}$ HFs exhibited expanded precortex and cortex when compared to wt HFs (Kaufman et al., 2003, Fig. 6D).

Similar to Krtap16-7, the expression domain detected with the AE13 antibody was not separate from the pigmented cells (Fig. 6D, arrowheads).

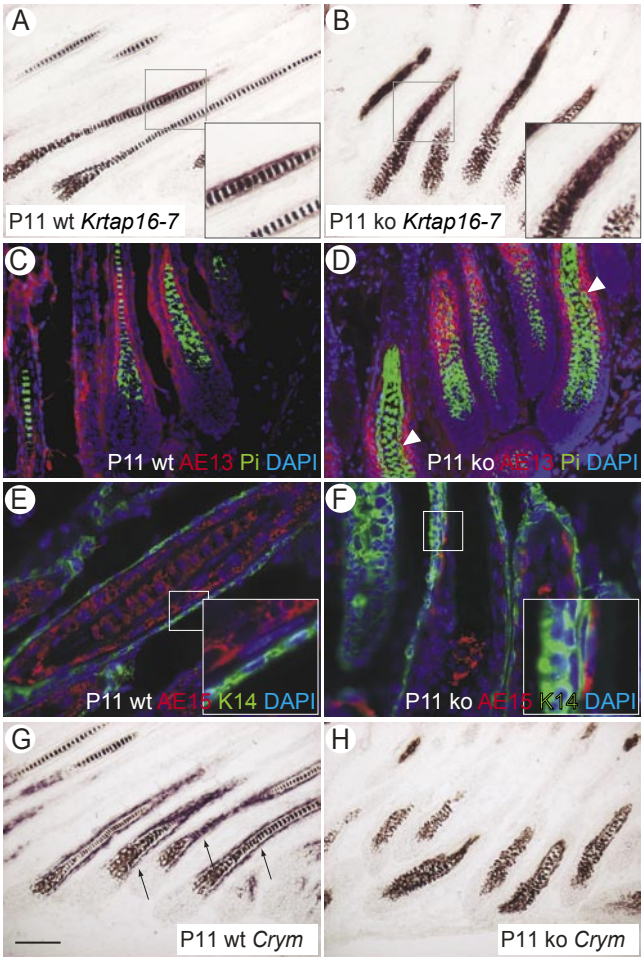


Figure 6. Hair follicle in the absence of Gata3. (A,B) In situ mRNA hybridization of Krtap16-7 at P11; insets show normal cortex expression in the wt (A), whereas expression spreads into the medulla in K14-Gata3$^{-/-}$ hairs (B). (C,D) Cortical cells recognized by AE13 antibody were no longer separate from the pigmented cells in K14-Gata3$^{-/-}$ HFs and those HFs exhibited expanded precortex and cortex as compared with wt HFs. Arrowheads indicate lack of separation of cortical cells from the pigmented cells. DAPI was used to mark the nuclei. (E,F) AE15 antibody recognizes trychohyalin IRS and medulla of control HFs (E) and only wisps of cells in K14-Gata3$^{-/-}$ IRS (F). There is a lack of separation of AE15-positive cells from K14-positive cells in K14-Gata3$^{-/-}$ HFs as compared with control (E,F, insets). (G,H) Expression of Crym at P11 in control (G) and K14-Gata3$^{-/-}$ skin (H); arrows in G point to expression in IRS. Scale bar in G: 200 μm for A,B,G,H; 100 μm for C,D; 50 μm for E,F.

AE15, an antibody that recognizes trychohyalin found in characteristic granules of the IRS and medulla (O'Guin et al., 1992 and Fig. 6E), detected a thin bundle of AE15 (+) K14-Gata3$^{-/-}$ IRS cells (Kaufman et al., 2003, Fig. 6F). Double staining with AE15 and K14 antibodies revealed that the companion layer did not separate AE15 (+) and K14 (+) cells (Fig. 6E, F and insets).

Consistent with the absence of Huxley's layer and the cuticle of the IRS in K14-Gata3$^{-/-}$ HFs, expression of μ-crystallin, known to co-localize with Gata3 in the IRS (Fig. 6G and Aoki et al., 2000), was diminished in K14-Gata3$^{-/-}$ HFs (Fig. 4H and 6H).

Next, we examined the differentiation status of K14-Gata3$^{-/-}$ mouse skin. Staining of loricrin, a late-stage differentiation marker, showed no differences in both the young (P1, Fig. 7A, B) and mature skin (5 months, Fig. 7C, D). In P4 skin, K10 expression was not different between the wt and K14-Gata3$^{-/-}$ mice (Fig. 7E, F).

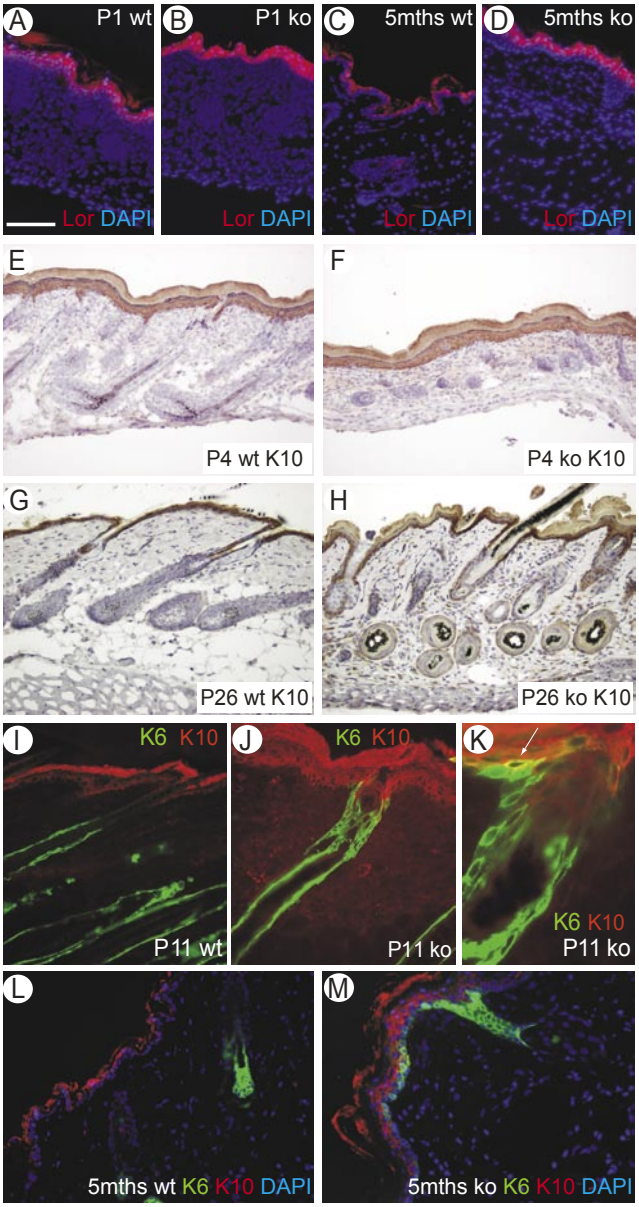


Figure 7. Skin localization of differentiation and proliferation markers in control and K14-Gata3$^{-/-}$ skin. (A-D) Normal staining of the late-stage differentiation marker loricrin at P1 (A,B) and 5 months (C,D). (E,F) Normal staining of the differentiation marker K10 at P4. (G,H) K10 expression at P26; note the cyst-like HFs in K14-Gata3$^{-/-}$. (I-M) Double-labelling of K10 (red) and K6 (green) at P11 (I-K) and at 5 months (L,M). There are clearly separated expression domains in the wt (I), but in K14-Gata3$^{-/-}$ K6 expression extends more distally (J); the arrow in K indicates a double-labelled (yellow) cell present in the K14-Gata3$^{-/-}$. Scale bar in A: 200 µm for A-H; 150 µm for I,J,L,M; 10 µm for K.

In P26, K10 was expressed in a layer surrounding the proximal part of K14-Gata3$^{-/-}$ HF, the ORS and the basal epidermal keratinocytes (Fig. 7H). In wt skin, however, it was restricted to suprabasal epidermal keratinocytes and infundibulum (Fig. 7G). K6, a cytokeratin known to mark proliferating cells proximal to the bulge and K10 was observed distal to the bulge and in the basal epidermis (Fig. 7I-K). Occasionally, K10 and K6 co-localized (Fig. 7K). At later stages the number of K6

expressing cells in the skin has increased substantially (Fig. 7L, M). Subsequent immunohistochemical analysis of K14, typically expressed in the ORS and the basal layer of stratified squamous epithelia of normal HFs (Vassar et al., 1989), confirmed our previous findings (Fig. 4H). Interestingly, the basal epidermal layer and ORS were almost twice as thick in the K14-Gata3$^{-/-}$ compared to wt skin (Fig. 6E, F). Together, these changes indicate aberrant differentiation, which is likely to be due to the deregulation of signal transduction pathways.

Signal transduction and embryonic development in K14-Gata3$^{-/-}$ HFs.
Most prominent signal transduction pathways known to be involved in HF morphogenesis were significantly overrepresented in the K14-Gata3$^{-/-}$ HF transcriptome including the Wnt and BMP families, Sonic hedgehog and Notch (Fig. 4G-H). Wnts 3, 10b, and 11 were upregulated in K14-Gata3$^{-/-}$ HFs, while Wnts 5a and 6 were downregulated compared to controls (Fig. 4H). Whereas in the wt HF, Wnt5a expression was detected in the ORS, IRS and dermal papilla (Fig. 8A), but was almost undetectable in K14-Gata3$^{-/-}$ HFs (Figs 4H and 8B), a finding that was in agreement with the absence of differentiated IRS cells, the significant decrease in shh mRNA levels (see below) and the known absence of Wnt5a expression in Shh$^{-/-}$ mice (Reddy et al., 2001). Wnt11 was previously shown to be expressed in the outermost layers of the HF, ORS and dermal sheath, above the dermal papilla (Reddy et al., 2001 and Fig. 8C). Similar to K10 (Fig. 7H), Wnt11 expression was expanded to the lowest (most proximal) part of the bulb in K14-Gata3$^{-/-}$ HFs (Fig. 8D) and was also present in the K14-Gata3$^{-/-}$ outer HF layer, as compared with controls. This could originate from an increased number of precursor IRS cells in the matrix that are in turn likely to be due to a migration defect that could also underlie the observed thickening of the matrix. Frizzled Wnt receptors Fzd1, Fzd3 and Fzd6 were overexpressed in the K14-Gata3$^{-/-}$ HF, whereas frizzled-related protein (Frzb, an inhibitor of Wnt signaling) was significantly downregulated (Fig. 4H). In agreement, we detected strong nuclear β-catenin staining in K14-Gata3$^{-/-}$ HFs (Fig. 8 E and F). Shh along with Gli1, which is a known target and transducer of Shh signaling, the hedgehoginteracting protein (Hhip) and patched homolog 2 (Ptch2) demonstrated reduced expression levels in K14-Gata3$^{-/-}$ HFs (Fig. 4H). The decrease in Gli1 expression levels was also confirmed by in situ hybridization in P7 K14-Gata3$^{-/-}$ HFs (Fig. 8G, H), particularly in the region of the dermal papilla.

Whereas Notch1 was upregulated in the K14-Gata3$^{-/-}$ HF (Fig. 4H), the expression levels of Notch2 and Notch3 were not significantly affected. Subsequent in situ mRNA hybridization showed Notch1 to be expressed in precursor cells of cortex, cuticle, and IRS (Fig. 8I and Kopan and Weintraub, 1993). Although the expression pattern of Notch1 appeared unchanged in the K14-Gata3$^{-/-}$ HF, its expression levels were substantially higher, particularly in the lower matrix cells (Fig. 8J). Taken together, these findings support the notion that Gata3 plays a central

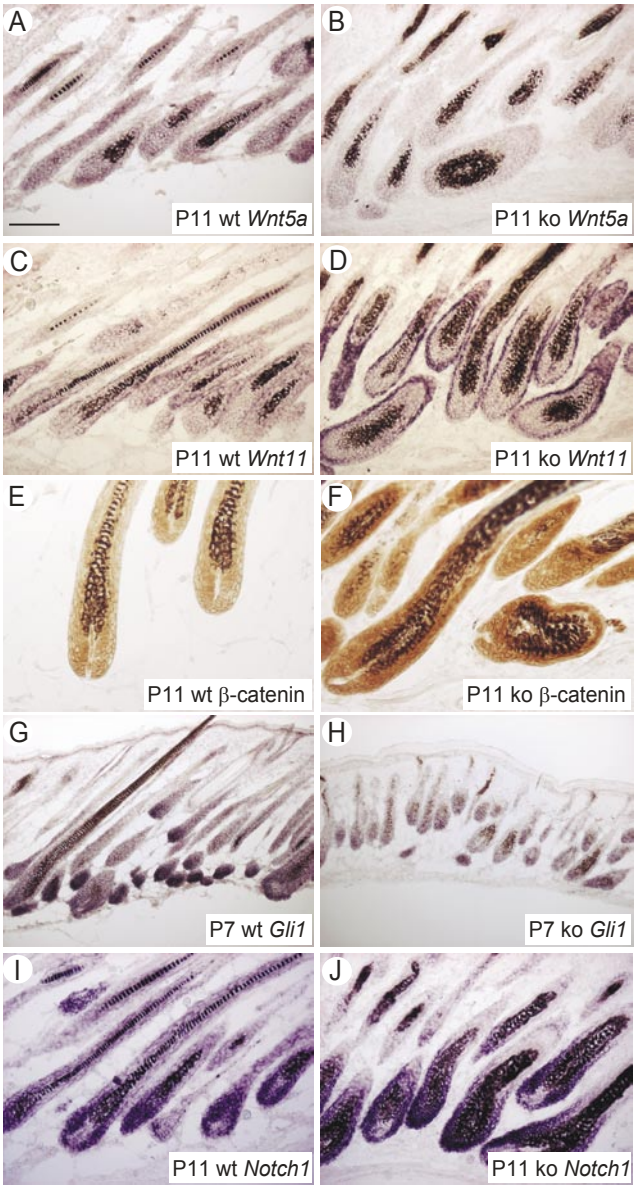


Figure 8. Expression pattern of signalling molecules in HFs in the absence of Gata3. (A,B) Reduced expression levels of Wnt5a in K14-Gata3$^{-/-}$ HFs (B) as compared with wt littermate controls (A). (C,D) Increased Wnt11 expression in the K14-Gata3$^{-/-}$ outer HF layer (D) as compared with wt controls (C) at P11. (E,F) Stronger nuclear ß-catenin staining in K14-Gata3$^{-/-}$ HFs in the matrix and in the distal part of the HF (F) as compared with control (E) at P11. (G,H) Decrease in Gli1 expression levels, particularly in the region of the dermal papilla in P7 K14-Gata3$^{-/-}$ HFs (H) as compared with controls (G). (I,J) Higher expression levels of Notch1 at P11 in the K14-Gata3$^{-/-}$ HF (J) as compared with wt (I). Scale bar in A: 150 μm for A-D,I,J; 100 μm for E,F; 200 μm for G,H.

role in Shh and Notch signaling pathways though this effect is likely indirect as shh and notch are expressed in different cells.

Three BMP genes (Bmp6, Bmp8a and Bmp15) were overexpressed in K14-Gata3$^{-/-}$ HFs as compared with wt controls; by contrast, BMP-receptor expression did not demonstrate significant changes. We examined BMP targets (Smad1/5/8-P and Id2) and an antagonist (gremlin) in the K14-Gata3$^{-/-}$ in greater detail (Supplementary Fig. S5). In situ hybridization showed slightly elevated expression

of Bpm6 in K14-Gata3$^{-/-}$ HFs compared to control (Supplementary Fig. S5A, B). Phosphorylation of Smad1/5/8 proteins was higher and more expanded in K14-Gata3$^{-/-}$ HFs, especially in the matrix (Supplementary Fig. S5D) and epidermis (Supplementary Fig. S5F). Interestingly, the expression of the BMP antagonist Gremlin that is expressed in the Henle's layer (Supplementary Fig. S5G) was absent in K14-Gata3$^{-/-}$ HFs (Supplementary Fig. S5H). Id2 protein (target for BMPs) was expressed in the cuticule of IRS (Supplementary Fig. S5I), epidermis and in the distal ORS (Supplementary Fig. S5K), but was absent in proximal K14-Gata3$^{-/-}$ HF (Supplementary Fig. S5J) while it was upregulated in epidermis and the distal part of HF in K14-Gata3$^{-/-}$ skin (Supplementary Fig. S5L).

The majority of transcription factors are upregulated in K14-Gata3$^{-/-}$ HFs.
Several transcription factors demonstrated deviant expression profiles in the K14-Gata3$^{-/-}$ HF (Supplementary table S1) although differences were smaller than those of structural genes. The majority of transcription factors were overexpressed in the K14-Gata3$^{-/-}$ HF (27 up, 12 down) indicating that Gata3 can function as a transcriptional repressor (and activator) in line with recent data showing that Gata factors interact with Fog1 (Zfpm1 - Mouse Genome Informatics) to form repressive chromatin complexes (Rodriguez et al., 2005). Cutl1 that encodes the transcriptional repressor CDP, known to be involved in epidermal differentiation, was downregulated in the K14-Gata3$^{-/-}$ mice (Fig. 4H). However, the hair phenotype of Cutl1$^{-/-}$ mice (e.g. circle hairs, corkscrew hairs) as well as several documented transcriptional changes are different to those of K14-Gata3$^{-/-}$ mice (Ellis et al., 2001) suggesting that CDP and Gata3 likely function in distinct signaling pathways.

Stem cell markers in K14-Gata3$^{-/-}$ skin
To analyze the presence and distribution of bulge cells in the K14-Gata3$^{-/-}$ skin and to delineate the role of Gata3 in the formation of the bulge, we investigated the expression of stem cell markers keratin 15 and S100A6 (Blanpain et al., 2004; Kizawa and Ito, 2005 and Cotsarelis, 2006). Compared to wt skin, keratin 15 and S100A6 were dramatically upregulated in the distal K14-Gata3$^{-/-}$ HF and were also present in the epidermis at P11, P19 and 5 months. Whereas the HF and epidermis expression domains are disconnected in wt, they form a continuum in the K14-Gata3$^{-/-}$ mice (Supplementary Fig. S6) suggesting that the bulge contributes more to the knockout epidermis and that Gata3 function is different in the epidermis than in the HF. As a result, the bulge also appears to contribute to epidermal thickening in addition to the increased proliferation of the epidermal cells (Fig. 5A, B). To obtain additional evidence for the contribution of the HF to the epidermis we examined the bald inside of the ear versus the outside, which does have many HF.

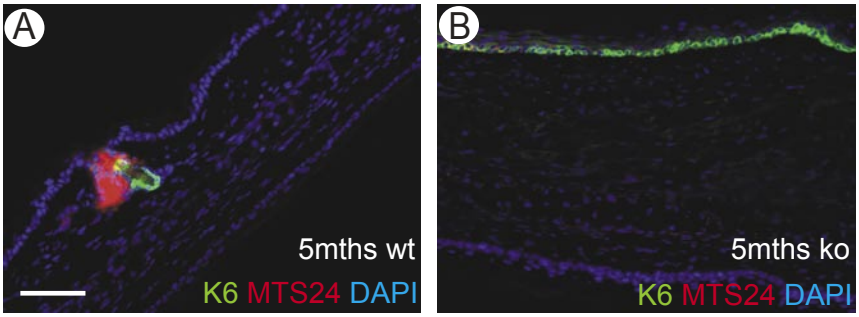


Figure 9. K6 expression pattern in the ear. Expression of K6 (green) versus MTS24 (red), which marks specific progenitor cells in the HF (Nijhof et al., 2006), at 5 months in wt (A) and K14-Gata3$^{-/-}$ (B) ears. The epidermis on the outside of the ear is towards the top of the image; the epidermis on the inside of the ear is towards the bottom of the image. Scale bar: 150 µm.

Interestingly Fig. 9A shows that K6 the marker for proliferating cells is absent in epidermis from both the inside and outside of the ear, whereas it is present in HF. In K14-Gata3$^{-/-}$ the epidermis on the outside ear expresses K6, whereas the inside that never had HF's, remains K6 negative (Fig. 9B).

Discussion

Gata3 is essential for proper maintenance of the epidermis and hair follicle cycling. Deletion of Gata3 gene in the murine epidermis and HF results in postnatal growth and developmental abnormalities along with aberrant hair growth, abnormal HF organization and pigmentation and pronounced epidermal thickening. This was followed by the gradual appearance of skin wrinkles, while one layer of the HF, the IRS, was not properly formed (see also Kaufman et al., 2003). In the present study, Gata3 is also expressed in layers other than the IRS of the HF, and all layers are disturbed. Normally, cells in the bulge reform the proximal part of the HF to replace shed hairs by new ones. This process does not take place in the K14-Gata3$^{-/-}$ knockout resulting in bald mice. Despite their disoriented appearance, the number of HFs was not substantially reduced, suggesting that Gata3 is not essential for the early steps of HF induction. Instead, Gata3 appears to have a role in hair cycling, as bulge cells no longer give rise to a new follicle. This is consistent with Gata3$^{-/-}$ skin grafting experiments showing that, in the absence of Gata3, the hair structures are still apparent in very early skin although with an aberrant morphology. The Gata3$^{-/-}$ skin was not analyzed at later stages in those transplantation experiments (Kaufman et al., 2003).

Gata3 impinges on the distribution of proliferating cells in the hair follicle matrix. Despite significant advances in our understanding of the role of Gata3 in HF development, little is known about those biological processes most significantly

affected in the absence of Gata3 in the murine epidermis and HFs. Therefore, we implemented a full mouse transcriptome analysis in laser-captured HFs to get an unbiased insight into (i) the implicated biological processes, (ii) the signalling mechanisms involved and (iii) the nature of the defect itself. This approach revealed several processes most pertinent to the cell cycle, epithelial growth and differentiation and signal transduction pathways. Importantly, we identified a broad, uniform decrease in the expression of most genes associated with the transition of the mitotic cell cycle. However, subsequent evaluation with BrdU staining revealed that, although the overall number of BrdU (+) cells did not differ between the K14-Gata3$^{-/-}$ and wt HFs, there was a significant difference in the distribution of proliferating cells throughout the HF. These findings highlight the direct role of Gata3 in differentially regulating specific cell lineages that originate from the matrix area and together with previous data (Kaufman et al., 2003) suggest that loss of Gata3 negatively impacts on proliferation and cell fate decisions in the matrix, likely shifting matrix cells towards fates other than the inner root sheath. Consistent with the decreased proliferative capacity of those cells located in the basal layer of the HF, apoptosis was reduced in the K14-Gata3$^{-/-}$ HF, likely reflecting the delayed onset of growth and differentiation. In agreement, several pro-apoptotic genes were downregulated while anti-apoptotic genes were upregulated. Coupled to this response, a number of genes involved in histone metabolism and chromatin modification were significantly upregulated in the absence of Gata3 from the HF.

Epithelial growth and differentiation represent significantly affected processes in the K14-Gata3$^{-/-}$ HF. Notably, changes in expression of genes encoding keratins and keratin-associated proteins were amongst the broadest identified. IRS cells express a number of genes that are also expressed in the suprabasal epidermal layers, suggesting that both cell types employ comparable differentiation pathways (Botchkarev and Paus, 2003). K10 expression is activated in terminally differentiated epidermal keratinocytes, when they start loosing their proliferative competence. Conversely, K10 expression is severely reduced under conditions that promote proliferation (Fuchs and Green, 1980; Fuchs and Weber, 1994; Moll et al., 1982), whereas ectopic K10 expression can induce an Rb mediated cell cycle arrest (Paramio et al., 1999; Santos et al., 2002). By contrast, K14-Gata3$^{-/-}$ mice express (ectopic) K10 in proliferating basal epidermal cells and in the decreased proliferating cells in the bulb of the HF. By analogy to Gata1 (Rodriguez et al., 2005), this suggests that Gata3 has (at least) a dual regulatory function affecting a number of genes involved in cell proliferation and terminal differentiation.

K10 expression is also regulated by the transcription factors C/EBP and AP-2 (Tcfap2a - Mouse Genome Informatics) (Maytin et al., 1999). Despite certain differences between the Cebpβ$^{-/-}$ and K14-Gata3$^{-/-}$ mice, some phenotypic parallels are striking. Whereas in Cebpβ$^{-/-}$ mice, the epidermis, the dermis and the size

and number of HFs appear normal, K10 expression is expanded, similar to the K14-Gata3$^{-/-}$ mice and subcutaneous fat is also decreased (Maytin et al., 1999). Interestingly, the two C/EBP-binding sites in the K10 (Krt10 - Mouse Genome Informatics) promoter flank a highly conserved GATA-binding site, suggesting that Gata3 and C/EBP regulate K10.

WNT and BMP signalling pathways in the K14-Gata3$^{-/-}$ hair follicle. Wnt signaling regulates HF development. Mice lacking Lef-1, a downstream Wnt mediator, demonstrate a reduced number of body hairs (van Genderen et al., 1994), whereas ectopic expression of either Lef-1 or constitutively active β-catenin induces ectopic HFs (Gat et al., 1998; Noramly et al., 1999; Zhou et al., 1995). Upon Wnt signaling, β-catenin accumulates in the cytoplasm and is transported to the nucleus, where it interacts with members of the LEF/TCF family of transcription factors, thereby activating the expression of downstream gene targets (Barker et al., 2000). K14-Gata3$^{-/-}$ show a considerable change in Wnt-related protein expression (see results and Figs 4 and 8E, F), which probably underlies the abnormal hair formation (DasGupta and Fuchs, 1999). Our result are in agreement with those obtained in Bmpr1a$^{-/-}$ mice, which have decreased level s of Gata3 (Andl et al., 2004; Kin Ming Kwan, 2004; Yuhki et al., 2004).

Interestingly, all BMP family genes whose expression changed significantly in the K14-Gata3$^{-/-}$ mice (BMP6, 8a, and 15) were upregulated (Fig. 5H). Moderately elevated BMP-6 signalling leads to increased proliferation of basal epidermal keratinocytes (Blessing et al., 1996; Botchkarev et al., 1999) similar to that observed in the K14-Gata3$^{-/-}$ basal epidermal layer. However, the phenotype of mice overexpressing BMP-6 in suprabasal epidermis (Blessing et al., 1996) is distinct from that seen in K14-Gata3$^{-/-}$ mice.

FGF and Notch1 signalling pathways in the K14-Gata3$^{-/-}$ hair follicle. Of all FGF family members (Supplementary Table S1), only Fgf5 was downregulated in K14-Gata3$^{-/-}$ mice, while Fgfr2 demonstrated increased mRNA expression levels. Mutation of Fgf5 (Hebert et al., 1994) or expression of a dominant negative Fgfr2 (Schlake, 2005) leads to the growth of long thin hair, while Fgfr2$^{-/-}$ mice (Petiot et al., 2003) have decreased number of HFs, impaired hair formation and reduced basal epidermal cell proliferation. Although, the number of HFs was not affected in K14-Gata3$^{-/-}$ mice, Fgf5 was downregulated, as opposed to increased expression of its receptor, suggesting that the receptor expression is likely to be rate-limiting. Lastly, Notch1 was upregulated in K14-Gata3$^{-/-}$ mice. As Notch1 expression, driven by involucrin in the IRS and suprabasal epidermis (Uyttendaele et al., 2004), provokes a similar, yet less severe hair phenotype, our findings highlight the possibility of Notch1 to directly affect hair formation.

In essence, several transgenic and/or knockout mouse models display phenotypic overlap (e.g. epidermal hyperplasia, reduced HF growth). In the majority of these cases, the hair phenotype is frequently accompanied by changes in adipogenesis and fat metabolism previously thought to be an indirect effect. In addition, increased or reduced expression levels for certain genes e.g. Hoxc13 (Godwin and Capecchi, 1998; Tkatchenko et al., 2001), BMPs (Blessing et al., 1996; Botchkarev et al., 2002), Notch1, (Uyttendaele et al., 2004; Vauclair et al., 2005) associated with the signaling mechanisms in hair formation appear to exert similar effects. However, for the majority of genes, it becomes increasingly apparent that their exact temporal and spatial expression is crucial; whereas reduction in Shh expression levels results in disturbed HF growth (St-Jacques et al., 1998), its increase leads to epidermal hyper-proliferation with distinct phenotypic features (Ellis et al., 2003).

Our results show that Gata3 is a key transcription factor that impinges on the regulation of several processes resulting in a composite of the phenotypes described above. The changes in keratin expression suggest that Gata3 acts as a moderator between HF development and epidermal differentiation through the orchestrated regulation of distinct signal transduction pathways. If Gata3 levels are greatly diminished, the epidermis hyperproliferates and HF matrix cells do not develop into functional IRS cells. Instead, the HF appears to contribute to the basal epidermis. This switch in mode of action has previously been proposed for Notch1 (Uyttendaele et al., 2004), a negative target of Gata3. In turn, BMPs regulate Gata3 (Andl et al., 2004; Kobielak et al., 2003) and vice versa (this paper) to maintain appropriate Gata3 expression levels. We, therefore, propose that Gata3 is a crucial component in the choice between forming different layers of the HF versus basal epidermal cells maintaining their balance through the coordinated regulation of the BMP and Notch signaling pathways.

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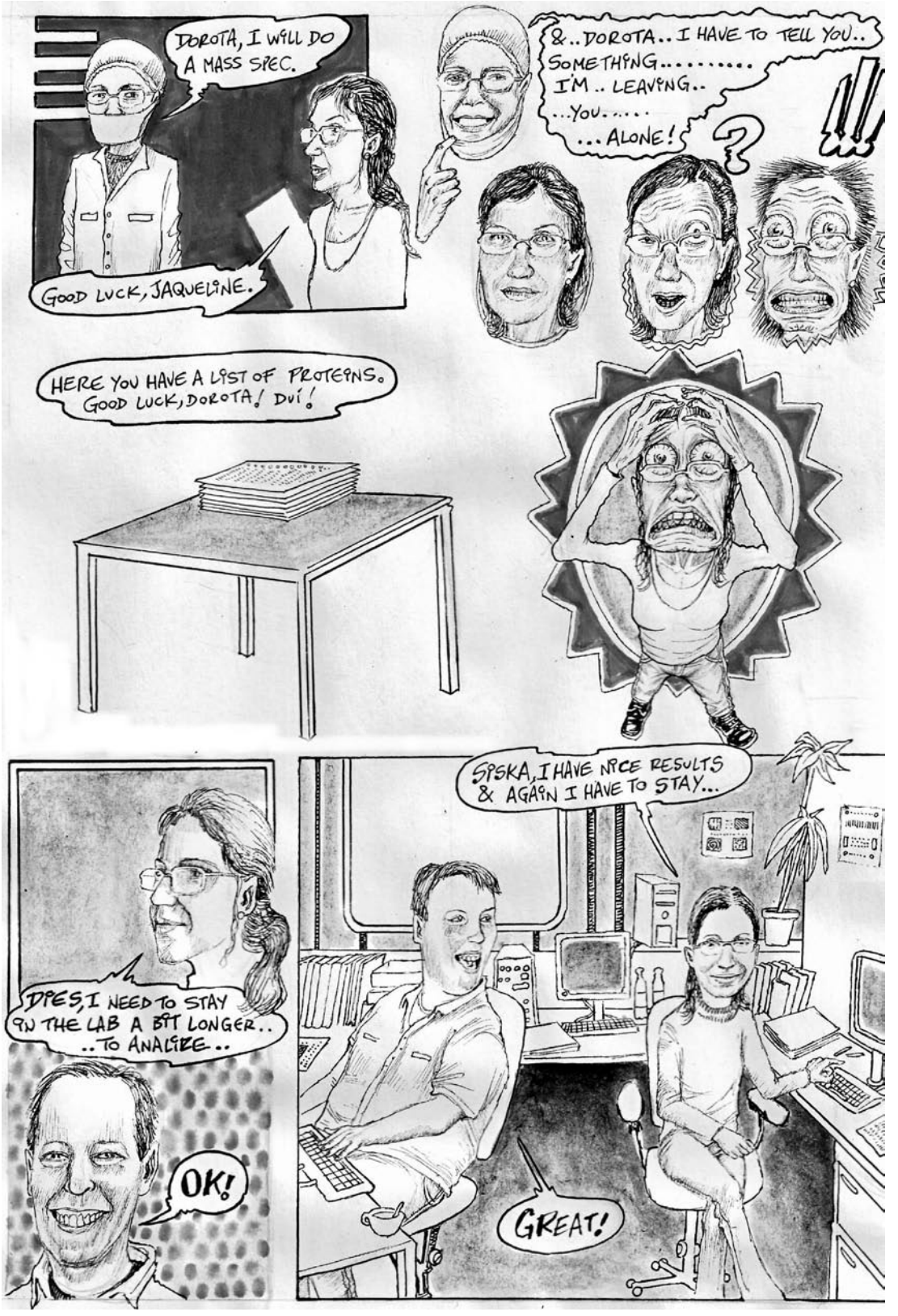
DOROTA, I WILL DO A MASS SPEC.
GOOD LUCK, JAQUELINE.
&..DOROTA..I HAVE TO TELL YOU..
SOMETHING.....
I'M .. LEAVING..
...YOU.....
...ALONE!
HERE YOU HAVE A LIST OF PROTEINS. GOOD LUCK, DOROTA! DUI!
SPSKA, I HAVE NICE RESULTS & AGAIN I HAVE TO STAY...
GREAT!
DIES, I NEED TO STAY ON THE LAB A BIT LONGER.. ..TO ANALIZE..
OK!

Chapter 3

Proteomic profiling identifies abnormal epidermal differentiation in the K14-Gata3-/- skin

Manuscript in preparation

Proteomic profiling identifies abnormal epidermal differentiation in the K14-Gata3$^{-/-}$ skin

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The epidermis of the skin is a highly specialized stratified epithelium that functions to protect the body from physical and chemical damage, infection, dehydration, and heat loss. To maintain this critical barrier, epithelial cells (keratinocytes) undergo a program of terminal differentiation, expressing a set of structural proteins which assemble a specialized protective barrier structure on their periphery termed the cornified cell envelope (CE). In order to function correctly, stratified epithelia have to maintain tight cell-cell adhesion in the living cells and retain the dead, keratinized squames as a protective sheath prior to being sloughed. Here we show that the transcription factor Gata3 is important for the balance between the process of cornification and desquamation in the skin. Using a proteomic approach we show that the phenotype of Gata3 deficient skin is the result of failure to maintain the balance between proliferation and shedding.

Key words: mouse, Gata3, epidermis, cell proliferation, differentiation, cornification, desquamation, keratinocytes

Introduction

Differentiation of epidermal keratinocytes is a tightly regulated process culminating in the conversion of viable epidermal keratinocytes of the sub-corneal layers into dead corneocytes of the stratum corneum (Byrne et al., 1994; Hardman et al., 1998). The differentiation steps that take place continuously within adult epidermis occur in the developing epidermis of the fetus in a synchronized manner. In the mouse the first suprabasal layer of the epidermis is formed between embryonic days E14.5 and E15.5 and the number of epidermal cell layers further increases during the following days. On day E18.5 the stratum corneum is established and the periderm, a superficial epithelial cell layer that functions as a diffusion barrier during earlier development, is shed (Bickenbach et al., 1995).

Cornification is an essential process during which the cornified layer is produced to protect the body from the environment and to prevent loss of body fluid (Kalinin et al., 2002; Madison, 2003). Formation of a cornified envelope, an insoluble structure beneath the plasma membrane, is a hallmark of cornification (Sun and Green, 1976). The cornified envelope is composed of many different proteins (Steinert and Marekov, 1995; Steven et al., 1990), including small proteins with short tandem repeats (involucrin, loricrin, and small proline-rich proteins) (Robinson et al.,

Steinert et al., 1998; Steinert and Marekov, 1997; Yaffe et al., 1993; Yoneda et al., 1992), fused-type S100 proteins (profilaggrin, hornerin, repetin, and trichohyalin) (Baden, 1994; Koch et al., 2000; Simon et al., 1996), cytokeratins (Ming et al., 1994), and other proteins (elafin, cystatin A, envoplakin, and desmosomal components) (DiColandrea et al., 2000; Lorand and Conrad, 1984; Nakane et al., 2002; Ruhrberg et al., 1997; Ruhrberg et al., 1996; Steinert and Marekov, 1995; Takahashi et al., 1992). These proteins are cross-linked through ε-(γ-glutamyl)–lysine bonds catalyzed by epidermal transglutaminases (Lorand and Conrad, 1984). The corneocytes remain attached to the underlying skin for some time in order to fulfil the barrier function of the skin. When the corneocytes are replaced by differentiating keratinocytes from the inward layers, the components of corneodesmosomes or desmosomes between the outermost corneocytes are proteolytically degraded by proteases. Finally, the detached cells are shed into the environment. Epidermal desquamation is the final event in a program of differentiation lasting approximately 4 weeks (Eckert et al., 2005; Milstone, 2004; Segre, 2006).

Loricrin

Loricrin is the major component of the cornified envelope in the epidermis (Steven and Steinert, 1994). Loricrin expression is induced by differentiation agents including calcium (DiSepio et al., 1995; Hohl et al., 1991) and is detected in granules in the granular layer (Ishida-Yamamoto et al., 1996; Ishida-Yamamoto et al., 1993; Steven et al., 1990). During terminal differentiation loricrin is released from the granules and cross-linked in the cornified envelope (Ishida-Yamamoto et al., 1999).

Fillagrin

Filaggrin is a protein which is incorporated into cornified envelope proteins (Simon et al., 1996; Steinert and Marekov, 1995). Filaggrin is is a histidine-rich basic protein belonging to the fused gene family, which plays unique pleiotropic roles in epidermal differentiation. During terminal differentiation of cornification, profilaggrin which comprises keratohyalin granules in the stratum granulosum, undergoes dephosphorylation and proteolysis to form filaggrin, which then promotes the aggregation of keratin intermediate filaments, resulting in the formation of disulfide bonds among them (Dale, 1977; Dale et al., 1978; Dale and Kam, 1993; Lonsdale-Eccles et al., 1984; Presland and Dale, 2000). Filaggrin is further processed by modification of some amino acid residues and degradation to free amino acids. Chemical modification of amino acids to hygroscopic compounds is thought to contribute to epidermal hydration and flexibility (Rawlings et al., 1994), whereas urocanic acid formation from histidine serves as a UV absorber (Scott, 1981).

Hornerin

The hornerin protein is very similar to profilaggrin in its structural features, expression profiles, intracellular localization, proteolytic processing, and possible involvement in epidermal differentiation of the granular layer. In accordance with this, hornerin was detected in the granular and cornified layers of the mature epidermis. In the granular cells of the epidermis, the hornerin protein was detected in keratohyalin granules together with profilaggrin. Hornerin protein was cleaved during the process of epidermal differentiation, indicating possible posttranslational proteolytic processing as is observed in profilaggrin. Differentiation of primary mouse epidermal keratinocytes with calcium results in the induction of hornerin. Hornerin is structurally as well as functionally most similar to profilaggrin among the family members and plays pleiotropic roles, including a role in cornification (Makino et al., 2001; Makino et al., 2003).

Caspase 14

Terminal epidermal keratinocytes differentiation has been suggested to involve members of the caspase family of proteases (Allombert-Blaise et al., 2003; Eckhart, Ban et al., 2000; Eckhart, Declercq et al., 2000; Lippens et al., 2003; Weil et al., 1999). Caspases are expressed in many different tissues; with the exception of caspase-14, they cleave substrate proteins at specific sequence motifs. Caspase-1 and its homologs caspase-4, -5, and -11 are implicated in the activation of interleukin-1β and interleukin-18 (Martinon and Tschopp, 2004). Caspase-2, -8, -9, -10, and -12 are initiator caspases of apoptosis and activate caspase-3, -6, and -7 that subsequently degrade proteins essential for cell survival (Degterev et al., 2003). Furthermore, caspases have been implicated in certain other forms of cell differentiation (Alam et al., 1999; De Maria et al., 1999; Kang et al., 2004; Salmena et al., 2003; Woo et al., 2003). Unlike other caspases, caspase-14 is expressed in a tissue-restricted manner with epidermis being the primary site of expression (Eckhart, Ban et al., 2000; Eckhart, Declercq et al., 2000; Kuechle et al., 2001; Lippens et al., 2003). Up-regulation of gene transcription leads to accumulation of procaspase-14 in terminally differentiated epidermal keratinocytes (Alibardi et al., 2005; Eckhart, Ban et al., 2000; Eckhart, Declercq et al., 2000; Lippens et al., 2003). In human skin, the entire pool of endogenous caspase-14 is cleaved in the final stages of epidermal keratinocytes differentiation as normal stratum corneum contains only the subunits of caspase-14 (Fischer et al., 2004). Although the substrate protein of caspase-14 in skin remains unknown, the localization and the timing of its expression and activation strongly suggest that it has a function in terminally differentiated epidermal keratinocytes. Indeed caspase-14-deficient mice show that its absence during cornification severely alters the biochemistry of this process during terminal differentiation of keratinocytes, reducing the efficacy of the barrier against UVB irradiation and water loss (Denecker et al., 2007).

Epidermal proteases and their inhibitors

Lysosomal cysteine proteinases of the papain family have long been considered enzymes with exclusive functions in terminal degradation of proteins in the lysosomal compartment (Barrett and Kirschke, 1981). This was suggested by their high abundance in lysosomes, ubiquitous expression in mammalian tissues, and by the observation that a substantial portion of intracellular protein degradation can be suppressed by protease inhibitors with broad specificity for cysteine proteases.

Cathepsin L (CTSL) is a lysosomal cysteine protease that has been shown to be involved in epidermal homeostasis. Expression of procathepsin L has been reported in normal epidermis, eccrine sweat glands, HFs, and blood vessels(Thewes et al., 1997). Mice lacking CTSL develop periodic hair loss with alteration of hair follicle morphogenesis and cycling as well as hyperplasia, acanthosis and hyperkeratosis of the epidermis The HFs cycle of mice lacking expression CTSL is abnormal. They exhibit a perturbed formation of the hair canal and high levels of keratinocyte and melanocyte apoptosis (Tobin et al., 2002).. Furthermore, the molecular defect of the spontaneous mouse mutant furless is identified as a mutation in the ctsl gene (Roth et al., 2000).

Cathepsin C was proposed to be essential for establishing and maintaining the structural organization the epidermis of the extremities and the integrity of the tissues surrounding the teeth, and may indirectly contribute to the processing of proteins such as keratins (Nuckolls and Slavkin, 1999). The aberrant differentiation of the junctional epithelium that binds the gingival to the tooth surface could possibly be responsible for weakening the mechanical barrier to periodontal pathogens (Toomes et al., 1999).

Stefin A2 (Cystatin A)

Cystatin A is a member of cystein proteinase inhibitors and is a potent inhibitor of cathepsins. Topical application of peptide protease inhibitors increases stratum corneum thickness in mice (Sato et al., 1998) In particular, cystatin A. It is abundantly expressed in keratinocytes of the cornified cell envelope, efficiently inhibits cathepsin B and suppresses UVB-induced apoptosis of keratinocytes by inhibiting caspase 3 activity.

Keratins

Keratinocytes produce large amount of keratins giving the keratinocytes their characteristic strength. The change of expression of keratins from the basal to the suprabasal layers is tightly regulated during epidermal differentiation. Keratins are structural proteins of epithelial cells that can be divided into two subgroups, type 1 and type 2 keratins, based on biochemical properties such as molecular weight and isoelectric point. In vitro and in vivo, type 1 and type 2 keratins polymerize to form hetero-polymeric intermediate filaments (Gu and Coulombe, 2007). Mitotically active keratinocytes of the basal layer of the epidermis express K5

and K14 (Byrne et al., 1994; Nelson and Sun, 1983). As cells differentiate, they often downregulate transcription of K5/K14 and induce new sets of differentiation-specific keratins which vary among stratified tissues (Fuchs and Green, 1980; Moll et al., 1982; Stellmach et al., 1991). K1 and K10 are synthesized only in post-mitotic, differentiating keratinocytes and are, consequently, found in the suprabasal layers of the mouse epidermis (Fuchs and Green, 1980; Roop, 1987; Schweizer et al., 1988). K6 and K16 are expressed in differentiating cells of the outer root sheath (ORS) of the hair follicle and the dorsal spikes of tongue (Moll et al., 1982; Rentrop et al., 1986). K6 and K16 are also unusual in that they are induced suprabasally under certain conditions that include epidermal hyperproliferation (Mansbridge and Knapp, 1987).

C/EBPβ

The C/EBP family of transcription factors is composed of at least five distinct members C/EBPα, C/EBPβ, C/EBPδ, C/EBPε, and Ig/EBP(C/EBPγ) belonging to the basic leucine zipper (bZIP) class of transcription factors (Cao et al., 1991). C/EBPα and C/EBPβ are expressed in human and mouse primary keratinocytes as well as in the human, mouse, and rat interfollicular epidermis (Wang et al., 1996). Within the mouse interfollicular epidermis, C/EBPα is expressed in the nuclei and cytoplasm of suprabasal keratinocytes and weakly expressed in a perinuclear manner in some basal keratinocytes. C/EBPβ expression is highly compartmentalized and is exclusive to the nuclei of cluster of suprabasal keratinocytes which is morphologically consistent with the differentiative column of the epidermal proliferative unit. In primary mouse keratinocytes, C/EBPβ expression is upregulated during calcium-induced growth arrest and squamous differentiation. C/EBPβ is involved in the regulation of the early stages of squamous differentiation of epidermal keratinocytes. Forced expression of C/EBPβ inhibits growth, induces K1 and K10 in keratinocytes, and has minimal effects on later-stage differentiation markers. C/EBPβ-deficient mouse skin revealed a hyperplastic epidermis and decreases in K1 and K10 expression with minimal differences in involucrin, loricrin, or K5 expression (Zhu et al., 1999).

Materials and Methods

Primary cell culture

Pregnant mothers were sacrificed by a cervical translocation and pups were isolated at E18.5 and sacrificed by ip injections with 50 µl of Nembutal. Tail and limbs were amputated and the skin was removed, stretched and flattened on a piece of Whatmann paper, dermis-side down in a petri dish. Skins were incubated in dispase solution (4mg/ml) in "KERA" (keratinocyte serum-free medium Keratinocyte-SFM (Gibco) supplemented with 10 ng/ml epidermal growth factor, 10^{-10}M cholera toxin and antibiotics) at 4°C for 7h. The dermis

was peeled off from the epidermis. The epidermis was cut is small pieces and incubated with 0.25% trypsin, 2.5mM EDTA in KERA for 40 min at 37°C. The cell suspension was neutralized by 10% FCS in KERA and filtered through a 40-µm cell strainer to take of tissue debris. The cells were centrifuged at 800 RPM for 10 min, washed and resuspended in KERA. Cells were seeded at density of 1 x 10^5 cells cm^{-2} on collagen coated dish or slide. The medium was changed every alternate day.

Keratinocytes differentiation
Cultured keratinocytes on a glass slide were differentiated by EGF and CT depletion and high calcium concentration (2 mmol/L) for 24h.
Protein electrophoresis
Hairs including hair follicles and squames were plucked, boiled for 30 min. in sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 0.56M ß-mercapto-ethanol, 0.005% bromophenol blue, 10% glycerol, 10 mM DTT) and the proteins separated by one dimensional gel electrophoresis in 1.5 mm thick 10% NuPAGE Novex 10% Bis-Tris acrylamide gels (200V, 1 hr, RT). Gels were stained with Novex Colloidal blue staining kit according to the manufacturer's instructions (Invitrogen). All solutions and workspaces were kept as clean to avoid keratin contamination.

Enzymatic digestion of proteins
Colloidal blue stained protein bands were excised manually with a scalpel and transferred onto a 96-well low protein binding microtiter plate (Nunc A/S, Roskide, Denmark). Each excised plug was washed with 100 µl milli-Q for 5 minutes with shaking (650 rpm, Eppendorf Geratebau GmbH, Hamburg, Germany). Gel plugs were de-stained with 0.4% (w/v) ammonium hydrogen carbonate (Sigma-Aldrich Chemie BV), 30% acetonitrile in water by incubating two times for 20 minutes at RT. After a short wash with Milli-Q, the gel fragments were dried in a rotary evaporator (Savant, Farmingdale, NY) for 30 minutes. Protein digestion was performed overnight at RT in 4 µl of 100 µg/ml sequencing grade-modified trypsin (Promega, Madison, WI).

MALDI-TOF peptide analysis
After the specific hydrolysis at the carboxylic sides of lysine and arginine residues by trypsin, 7 µl (1:2) acetonitrile:0.1% trifluoroacetic acid was added to the gel plugs. After mixing, 1 µl of the tryptic digest was taken and co-crystallized with 2.5 µl 2 mg/ml of the photoactive compound α-cyano-4-hydroxy-trans-cinnamic acid (α-HCCA, Bruker Daltonics, Billerica, MA) in acetonitrile. This sample-matrix solution (0.5 µl) was pipetted onto a 400-µm 384-well anchor chip MALDI-TOF plate and air-dried for 5 minutes. Peptide mass spectra were acquired on a Biflex III MALDI-TOF mass spectrometer equipped with a 337-nm nitrogen laser (Bruker Daltonics, Bremen, Germany). The instrument was calibrated with a peptide calibration standard (Bruker Daltonics). Spectra were compared using autolytic fragments from trypsin. A mass list of peptides was obtained from each digest and submitted to Matrix Science Mascot UK software to identify the proteins in the MSDB database of the NCBI. The criteria for identification of proteins were determined as follows: maximum allowed peptide mass error of 200 ppm, at least five matching peptide masses, molecular weight of identified protein should match estimated values by comparing with marker proteins, and top scores given by software higher than 61 ($P < 0.05$).

Western Blotting
For Western blotting following one-dimensional SDS-PAGE, proteins were electroblotted onto Immobilon P membranes (Millipore Corp, Etten-Leur, The Netherlands) and incubated with specific antibodies, followed by horseradish peroxidase-conjugated secondary antibodies, and detected using SuperSignal West Pico chemiluminescent substrate (Pierce Perbio Science, Etten-Leur, The Netherlands). Antibodies used in this study to confirm the proteins detected by MALDI-TOF were caspase14.

Skin barrier
Experiments were performed as described by (Hardman et al., 1998).

Histology
Tissues were collected in Tissue-Tec embedding medium (Sakura) and snap-frozen in an ethanol-dry ice bath. 6 μm thick sections were cut, fixed in 4% paraformaldehyde and stained with hematoxylin and eosin.

In situ hybridization
In situ hybridization with antisense RNA probes labelled with digoxigenin-UTP was done according to (Schaeren-Wiemers and Gerfin-Moser, 1993) on 10μm thick sections of Tissue-Tec embedded snap-frozen P7 and P11 dorsal skin samples. Probe templates were suitably linearised mouse cDNAs of S100A3 (nt 155-51,NM011310), Dstn (nt 10-460, NP_062745), Ctsh (nt 411-1091, NM_007801), Stfa1 (nt 13-314, NP_001001332), Hrnr (nt 5-409, XR_004824), Ctsl (nt 112-764, NM_009984), Ctsc (nt 395-1078, NM_009982), Casp14 (nt 648-1520, NM_009809).

Immunohistochemistry and BrdU labelling. Mice were injected with 50mg/kg bodyweight 5-bromo-2'-deoxyuridine (BrdU) and sacrificed 2 hours later. Cells were washed with PBS. Cryosections and cells were fixed for 10 min in 4% PFA in PBS and washed three times for 5 min in PBS. As block/diluent was used: 1% BSA, 0.05% Tween in PBS. Primary antibodies that were used at the indicated dilutions were: BrdU (mouse 1:100; DAKO, clone Bu20a), and K10 (mouse 1:50; Sigma, clone k8.60), p63 (1:500 mouse, clone: 4A4, DAKO), loricrin (1:500 rabbit, Covance, PRB-145P), filaggrin (1:100 rabbit; M-290, sc-30230, Santa Cruz), C/EBPb (1:100 rabbit; sc-150, C19 Santa Cruz). Relevant FITC-, TxR- or HRP-conjugated goat antibodies (1:100, DAKO) were used to detect primary antibodies. When murine primary antibodies were applied, MOM blocking reagent (Vector Laboratories) was used to block endogenous immunoglobulins. For BrdU immunohistochemistry, tissue samples were fixed in 4% PFA in PBS at 4°C overnight. Skin samples were subsequently embedded in paraffin and sectioned at 5 μm. After deparaffination sections were boiled in 0.01M citrate buffer (pH 6.0) for 15 min. prior to incubation with primary antibody. Sections were studied and photographed with an Olympus BX40 light microscope. All fluorescent images were taken with an Axio Imager (Zeiss) fluorescence microscope

Results

Epidermal hyperplasia and hyperkeratosis in K14-Gata3$^{-/-}$ skin

Gata3 deletion in the inter-follicular keratinocytes results in hyperplasia and hyperkeratosis of the epidermis (Kurek et al., 2007).

K14-Gata3$^{-/-}$ fetuses had a slightly delayed barrier function at E17.5, E18.5 (not shown) and P1 (Fig. 1).

Figure 1. Dye exclusion assay performed on E18.5 embryos. K14-Gata3$^{-/-}$ fetuses (B) had a slightly delayed barrier function compare to control (A).

Initial development of the epidermis appeared normal in skin sections from newborn mice but later skin hyperplasia was followed by wrinkles covered with squames. K14-Gata3$^{-/-}$ skin sections, at different ages, revealed the gradual, pronounced epidermal thickening including that of basal, suprabasal and granular layers as well as the development of hyperkeratosis (increased thickness of stratum corneum) (Fig.2 A-E). K14-Gata3$^{-/-}$ epidermis demonstrated an elevated proliferation in the basal layer, shown by BrdU incorporation (Fig. 2D, E and Kurek et al., 2007). Increased thickening of the cornified layer prompted us to examine the differentiation status of K14-Gata3$^{-/-}$ mouse skin. Staining of loricrin, a late-stage differentiation marker, showed no difference at P3 (Fig. 3A, B) and the increased thickness of the loricrine positive layer in mature skin (Fig. 3C, D) concomitant with increased thickness of the cornified envelope. Staining for filaggrin, another differentiation marker, showed similar results (Fig. 3E-H).

p63 is critically involved in the regulation of epidermal differentiation. It initiates stratification during development and also maintains the proliferative potential of basal keratinocytes in the mature epidermis (King and Weinberg, 2007; Koster and Roop, 2004). The highest level of expression of p63 can be detected in the basal layer of the epidermis, and its downregulation in the differentiated layers of the epidermis is required for normal terminal differentiation. In the K14-Gata3$^{-/-}$ mouse skin an increase of p63-positive cells was observed (Fig. 3I, J). Interestingly, p63-positive cells were also found in the suprabasal layer of K14-Gata3$^{-/-}$ mouse skin (Fig. 3J, arrows). The persistence of p63 in the suprabasal layer suggests that epidermal stratification is delayed in the K14-Gata3$^{-/-}$ skin.

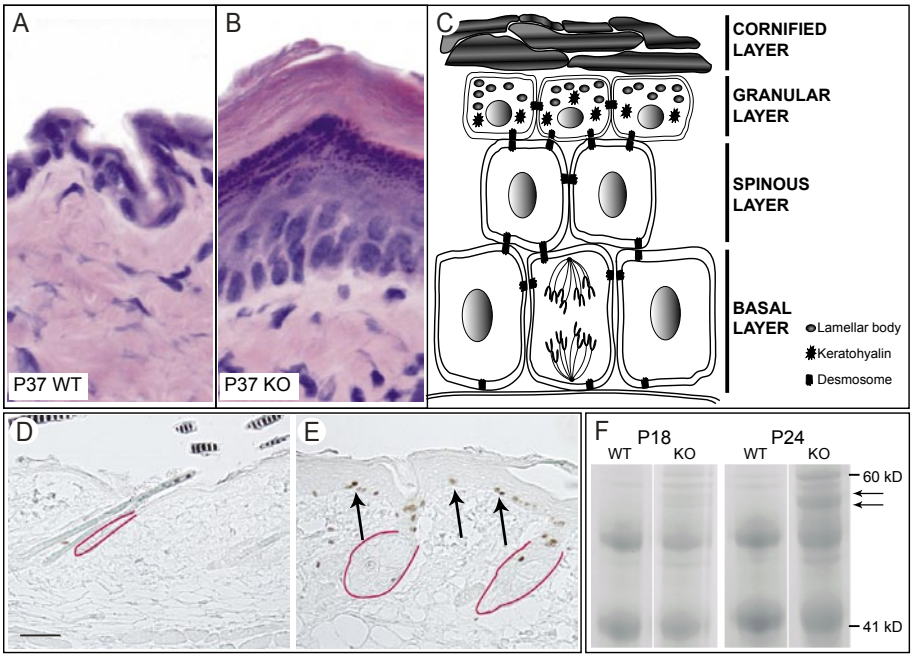

Figure 2. Haematoxylin-eosin staining of fresh-frozen skin sections of control (A) and K14-Gata3$^{-/-}$ (B) mice at P37. Basal, suprabasal and corneal layers are much thicker in the K14-Gata3$^{-/-}$ skin (B), compare to control skin (A) at P37. Schematic representation of the stages of epidermal differentiation. Epidermal keratinocytes undergo a linear program of differentiation from mitotically active basal cells to transcriptionally active spinous cells to enucleated granular cells, resulting finally in differentiated squames in the stratum corneum (C). BrdU labelling (brown stained nuclei) of dividing cells of control (D) and K14-Gata3$^{-/-}$ (E) epidermis at 5 months of age. The cells of the basal layer of the K14-Gata3$^{-/-}$ epidermis (E) are dividing at a very high rate compare to control (D). Sebaceous glands are enlarged in K14-Gata3$^{-/-}$ epidermis (encircled in red) and also contain mitotically active cells (E). Coomassie staining of the protein gel showing two extra protein bands (arrows) with an approximate weight of 53 kD (P18 and P24), shown in F. Scale bar in D: 10 µm for A and B; 150 µm for D and E.

Proteomic profiling of epidermal differentiation in the Gata3ko skin

In order to gain insight into the molecular mechanism of the thickened epidermis of the K14-Gata3$^{-/-}$ mice, we analyzed the protein composition of the hairs, their follicles and surrounding squames at P18 (hairs of control mice are in the catagen stage, but in the K14-Gata3$^{-/-}$ mice the hairs are in anagen), at P24 (normal hairs in telogen, K14-Gata3$^{-/-}$ hairs in catagen), and at 4 weeks of age (mixed developmental hair stages). Hairs were plucked at the indicated ages, boiled in sample buffer and proteins were separated on a 10% acrylamide gel. All hair samples of K14-Gata3$^{-/-}$ mice showed two extra protein bands with an approximate weight of 53 kD which were already visible by Coomassie staining (P18 and P24 are shown in Fig.2F). These bands were excised from the gel and analyzed by MALDI-TOF mass spectrometry. Table 1 shows a list of proteins, which appeared uniquely for wt or K14-Gata3$^{-/-}$ in the mixture of proteins present in these two fragments excised from the gel.

Proteins unique in control	Symbol	Score
cathepsin H	Ctsh	120
desmocollin 2	Dsc2	119
thioredoxin 1]	Txn1	112
dnaK-type molecular chaperone HSP70.2	HSP70	112
corneodesmosin	Cdsn	109
profilin 1	Pfn1	109
annexin A5	Anxa5	109
cysteine-rich secretory protein 2	Crisp2	109
heat shock protein 90kDa beta (Grp94)	Hsp90b1	107
S100 calcium binding protein A6	S100a6	107
proteasomesubunit, beta type 4	Psmb4	98
S100 calcium binding protein A3	S100a3	97
desmocollin 3	Dsc3	96
T complex protein 10	Tcp10b	94
trypsin 1	Prss1	93
keratin associated protein 3-1	Krtap3-1	93
keratin 42	Krt42	90
keratin associated protein 6-2	Krtap6-2	90
phospholipase A2, group IIE	Pla2g2e	89
hephaestin-like 1	Hephl1	87
keratin associated protein 21-1	Krtap21-1	85
lactate dehydrogenase B	Ldhb	85
histone 1, H4j	Hist1h4j	80
annexin A1]	Anxa1	80
cysteine-rich secretory protein 1	Crisp1	80
tubulin, alpha 7	Tuba7	77
katanin p60 subunit A-like 2	Katnal2	75
ADP-ribosylation factor 2	Arf2	74
leucine rich repeat containing 20	Lrrc20	73
integrin beta 4 binding protein	Itgb4bp	73
complement component 1, q	C1qbp	67
transglutaminase 3, E polypeptide	Tgm3	67
deoxyribonuclease 1-like 2	Dnase1l2	63
phosphoglycerate mutase 1	Pgam1	61
eukaryotic translation elongation factor 2	Eef2	61
lysosomal membrane glycoprotein 2	Lamp2	61
Proteins unique in Gata3 ko		
caspase 14	Casp14	150
keratin 10	Krt10	145
destrin	Dstn	132
cathepsin L	Ctsl	125
hornerin	Hrnr	124
glutathione S-transferase omega 1	Gsto1	119
stefin A1	Stfa1	119
ribosomal protein L5	Rpl5	119
cathepsin C	Ctsc	119
moesin	Msn	119
cofilin 1, non-muscle	Cfl1	119
proteasome 26S ATPase subunit 4	Psmc4	116
keratin associated protein 13	Krtap13	115

Proteins unique in Gata3 ko	Symbol	Score
Elongation factor 1-alpha 1	Eef1a1	115
histone 1, H1e	Hist1h1e	112
bromodomain-containing	Brd3	112
ADP-ribosylation factor 5	Arf5	112
heat shock-related protein	Hspa1l	112
actinin, alpha 1	Actn1	109
proteasome beta 3 subunit	Psmb3	108
3-hydroxybutyrate dehydrogenase, type 1	Bdh1	107
kallikrein 10	Klk10	107
alpha-actinin 2	Actn2	107
keratin associated protein 8-1	Krtap8-1	101
major urinary protein 2	Mup2	101
mouse preprocathepsin B	Ctsb	101
kallikrein 6	Klk6	99
keratin 28	Krt28	98
cadherin 8	Cdh8	96
ribosomal protein S14	Rps14	96
myosin, heavy polypeptide 10	Myh10	94
keratin 5	Krt5	94
phosphoglycerate mutase 2	Pgam2	94
polyubiquitin (clone arf3)	polyubiquitin	94
uvomorulin (711 AA)	Cdh1	94
annexin A1	Anxa1	90
ribosomal protein S24	Rps24	87
orphan short chain dehydrogenase	Sdro	86
Krt79 protein	Krt79	86
ribosomal protein L4	Rpl4	86
ribosomal protein S16	Rps16	86
clathrin, heavy polypeptide	Cltc	85
plectin 1	Plec1	80
Plastin-2	Lcp1	80
serine/arginine repetitive matrix 2	Srrm2	79
ribosomal protein L27a	Rpl27a	79
heterogeneous nuclear ribonucleoprotein A3	Hnrpa3	78
peroxiredoxin 1	Prdx1	78
keratin complex 2, basic gene 18	Krt85	74
neurofilament-L	Nefl	74
keratin 28	Krt28	73
myosin, heavy polypeptide 14	Myh14	68
coiled-coil domain containing 25	Ccdc25	68
chaperonin subunit 6a (zeta)	Cct6a	68
Malate dehydrogenase	Mdh1	68
lamin A	Lmna	65
heterogeneous nuclear ribonucleoprotein M	Hnrpm	63
keratin 85	Krt85	63
keratin 77	Krt77	62
catenin	Ctnnd1	62
ribosomal protein L23	Rpl23	62
RuvB-like protein 1	Ruvbl1	61
chaperonin subunit 5 (epsilon)	Cct5	61

Table 1. List of proteins, which appeared uniquely for control or K14-Gata3$^{-/-}$ in the mixture of proteins present in fragments excised from the gel.

Altered expression of differentiation markers

Keratin 1 and 10 were easily detectable only in K14-Gata3$^{-/-}$ mice, not in heterozygous mice. We investigated by immunohistochemistry the expression of these two cytokeratins that are normally present as a pair in the suprabasal differentiating layer in post-mitotic cells of the epidermis of cornifying epithelia, and that are absent from the proliferating basal layer and ORS. No differences in morphology or in expression of keratin1/10 proteins were observed in young wt and K14-Gata3$^{-/-}$ skin (Kurek et al., 2007). However, the adult K14-Gata3$^{-/-}$ mice had much thicker epidermis than wild-type epidermis (especially the granular layer and stratum corneum). A striking difference in expression was found in the lower

parts of the hair follicles: whereas in wild-type mice K1/K10 expression was absent from this region, the K14-Gata3$^{-/-}$ mice had high K1/K10 expression in the bottom area of the hair follicles (Kurek et al., 2007).

The staining of K1/10 antibody was present in differentiating layers of the epidermis in both wt and K14-Gata3$^{-/-}$ sections (Fig. 4A, B), however in the K14-Gata3$^{-/-}$ epidermis there was an additional expression of K1/10 in some cells of the basal layer of the epidermis (Fig. 4B, arrows).

The transcription factor C/EBPβ, which is involved in the regulation of the early stages of squamous differentiation of epidermal keratinocytes, regulates K1/10 expression in the epidermis. In the wt epidermis C/EBPβ expression appears in the basal layer (Fig.4 C, (Zhu et al., 1999) and in the K14-Gata3$^{-/-}$ skin C/EBPβ positive nuclei were found in the in all layers of the thickened epidermis with the strongest expression in the granular layer (Fig. 4D).

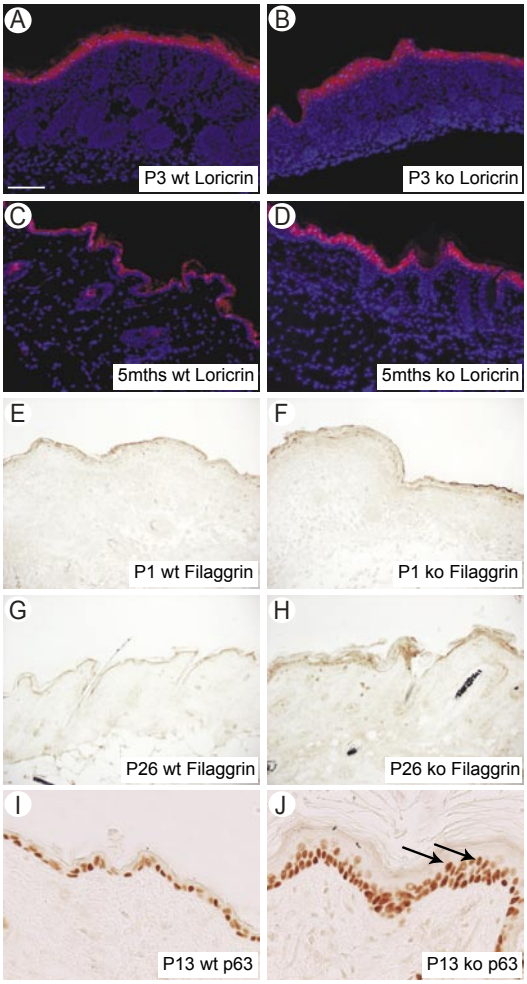

Figure 3. Epidermal localization of differentiation markers in control and K14-Gata3$^{-/-}$ skin. Normal staining of the late-stage differentiation marker loricrin in P3 (A-B) and increased thickness of loricrine positive layer in mature K14-Gata3$^{-/-}$ skin (C-D); E, F: Normal staining of the differentiation marker filaggrin G, H: Filaggrin staining at P26 shows increased thickness of granular and cornified layer. I, J: p63 staining shows the increased thickness of basal layer in K14-Gata3$^{-/-}$ skin (J). In contrast to control skin p63-positive cells are present in the suprabasal layer of K14-Gata3$^{-/-}$ mouse skin (J, arrows) Scale bar in A 200 µm for A-D and G-H; 150 µm for E and F; 50 µm for I and J.

We found hornerin protein to be uniquely present in the K14-Gata3$^{-/-}$ skin samples (Table 1). Hornerin, a protein very similar to profilaggrin, is expressed in the granular layer and is detected in the granular and cornified layers of the mature epidermis. At P13 hornerin was found in the granular layer of the epidermis of the wt animals and upregulated in the K14-Gata3$^{-/-}$ skin (Fig.4 E, F). In the wt adult epidermis expression of hornerin was downregulated (Fig. 4G) whereas expression in the K14-Gata3$^{-/-}$ skin increased (Fig. 4H)

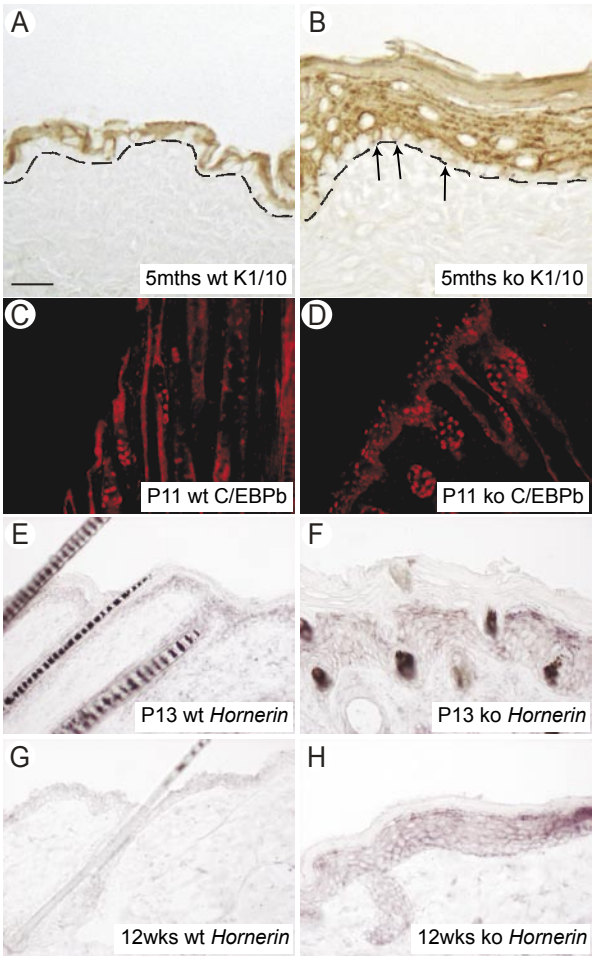

Figure 4. Expression pattern of differentiation molecules in epidermis in the absence of Gata3. K1/10 antibody staining present in differentiating layers of the epidermis in both wt and K14-Gata3$^{-/-}$ sections (A, B) and additional expression of K1/10 in some cells of the basal layer of the K14-Gata3$^{-/-}$ epidermis (B, arrows). C/EBPb expression in the basal layer of wt epidermis (C) and in the K14-Gata3$^{-/-}$ skin C/EBPb positive nuclei found in all layers of the thickened epidermis with the strongest expression in the granular layer (D). E-H: Hornerin expression was upregulated in the K14-Gata3$^{-/-}$ skin. Scale bar in A 25 µm for A and B; 150 µm for C and D; 50 µm for E-H.

Caspase14 is upregulated in the K14-Gata3$^{-/-}$ epidermis

Caspase 14 showed up as a unique protein in the sample isolated from K14-Gata3$^{-/-}$ animal. Unlike other caspases, caspase-14 is expressed in a tissue-restricted manner with epidermis being the primary site of expression (Eckhart, Declercq et., 2000;

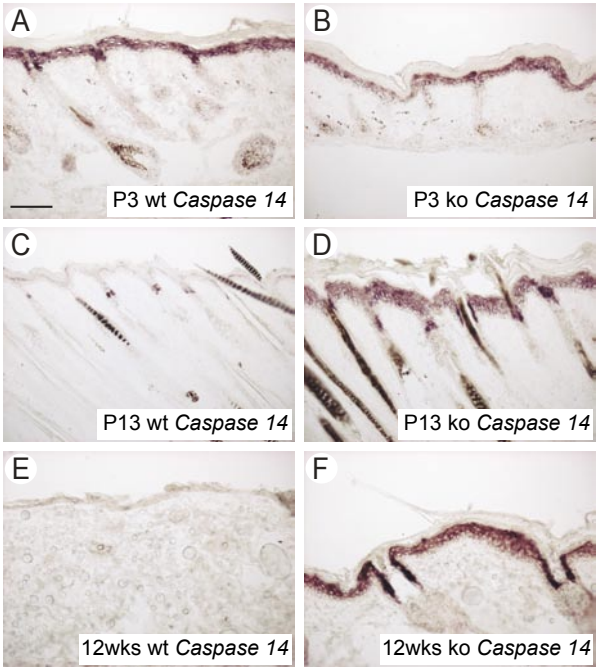

Figure 5. Upregulation of caspase 14 in the K14-Gata3$^{-/-}$ epidermis. At P1 caspase-14 expression in K14-Gata3$^{-/-}$ skin is similar to wild-type skin (A, B). Caspase 14 expression is downregulated in the epidermis of the wildtype mice (C, E) but upregulated in the K14-Gata3$^{-/-}$ (E, F). Scale bar in A 150 μm.

Kuechle et al., 2001; Lippens et al., 2003). Up-regulation of gene transcription leads to accumulation of procaspase-14 in terminally differentiated epidermal keratinocytes (Alibardi et al., 2005; Eckhart, Declercq et al., 2000; Lippens et al., 2003). Caspase-14 expression appears at the same time that murine skin begins to fully differentiate into a cornified epithelium, and is not expressed in noncornified epithelia. At P3, caspase 14 is expressed in the infundibulum and the suprabasal layer of the epidermis in both wild-type and K14-Gata3$^{-/-}$ skin (Fig.5 A, B). At this age, caspase-14 expression in K14-Gata3$^{-/-}$ skin is similar in abundance and expression pattern to wild-type skin, consistent with northern analysis (not shown). As postnatal development proceeds, caspase 14 expression is downregulated in the interfollicular epidermis in wildtype mice (Fig.5C, E). As K14-Gata3$^{-/-}$ skin matures, caspase14 expression in the infundibulum not only persists but increases (Fig.5D, F). Increased gene expression occurs before the morphologically distinct thickening of the epidermis can be identified, indicating that the upregulation is probably a cause rather than a consequence of thickening. At 12 weeks of age expression of caspase 14 in wt epidermis is not detectable by in situ hybridization but in the K14-Gata3$^{-/-}$ epidermis and expression is high in infundibulum (Fig.5 E, F).

Altered terminal differentiation of primary K14-Gata3$^{-/-}$ keratinocytes

We investigated the in vitro differentiation of primary keratinocytes isolated from wild-type and K14-Gata3$^{-/-}$ mice. In wild-type cultures the early marker of terminal differentiation K1/10 was induced by calcium and hardly present in the undifferentiated culture (Fig.8). In K14-Gata3$^{-/-}$ keratinocytes the terminal differentiation program was altered as an early differentiation marker K1/10 was already present in the undifferentiated culture and additionally upregulated during calcium induction. The late marker loricrin was upregulated in the undifferentiated K14-Gata3$^{-/-}$ keratinocytes, but surprisingly its expression did not change upon calcium induction. When keratinocytes commit to the terminal differentiation programme they activate early features of the differentiation program (K1/10-expression) followed by the presence of late markers (loricrin). Thus the K14-Gata3$^{-/-}$ keratinocytes appear to remain longer in the early differentiation state when compared to the control, which could explain the increased thickness of cornified layer in the K14-Gata3$^{-/-}$ mice.

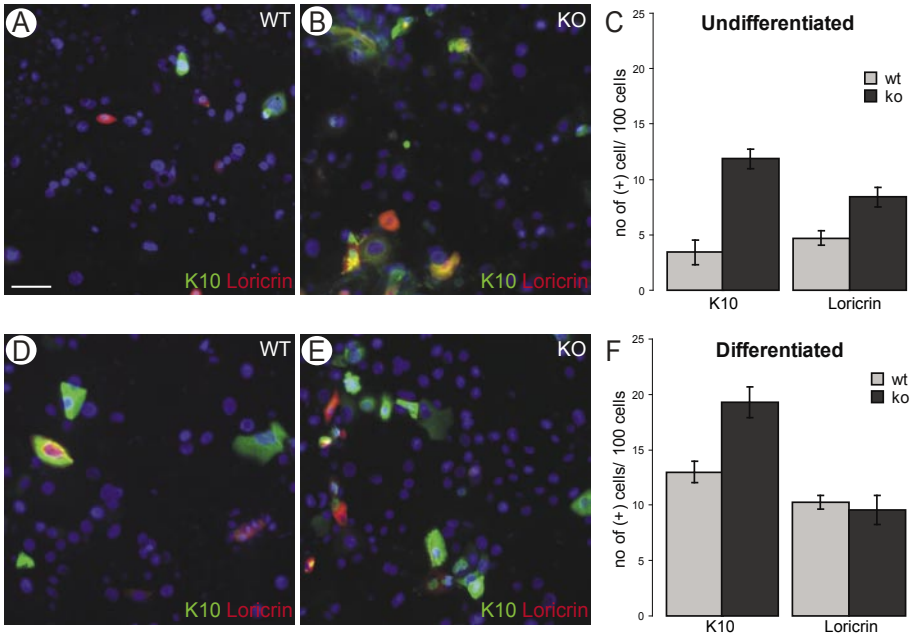

Figure 8. Terminal differentiation of primary K14-Gata3$^{-/-}$ keratinocytes. Upregulation of K1/10 expression in K14-Gata3$^{-/-}$ keratinocytes in uninduced and induced culture (A-F). Expression of loricrin is upregulated in undifferentiated K14-Gata3$^{-/-}$ keratinocytes (A-C) but the same in induced cultures (D-F). Scale bar in A 20 µm for A-B and D-E.

Discussion

Gata3 deletion in the inter-follicular keratinocytes results in hyperplasia and hyperkeratosis of the epidermis. Histochemical analysis demonstrates that the epidermal hyperplasia corresponds to an increase of the differentiating (K10-, caspase14-, hornerin- and filaggrin-positive) cell compartment of the epidermis, and the proliferative cell compartment of the epidermis (K6-, p63- and BrdU-positive). The increase in the differentiated cell compartment is both due to an increase in the suprabasal keratinocyte population (K1-positive, loricrin-negative) as well as the more terminal differentiated keratinocyte population (K1- and loricrin-positive). Histological analysis revealed that the basal keratinocytes which lack Gata3 undergo (some) premature differentiation as indicated by the appearance of K1/10 positive cells in the basal layer compartment and p63-positive cells in the suprabasal layer. The terminal epidermal differentiation program appeared to be unaltered, as loricrin expression is observed in K14-Gata3$^{-/-}$ mice.

Many of the misregulated proteins detected in the mass spec analysis are involved in keratinocyte terminal differentiation (K1/10, caspase14, hornerin), consistent with the role of Gata3 in the epidermis. One of the proteins with the highest score, caspase 14, is a member of the caspase family of proteins and is the only caspase with a tissue-restricted expression pattern (Ahmad et al., 1998; Eckhart, Declercq et al., 2000; Hu et al., 1998; Van de Craen et al., 1998). Although caspase 14 does not cleave classical caspase substrates and is not activated by apoptosis-inducing agents, the protein is processed during epidermal differentiation. The processing is associated with terminal keratinocyte differentiation (Eckhart, Declercq et al., 2000; Lippens et al., 2003), since caspase-14-deficient mice show that the absence of caspase-14 during cornification severely alters the biochemistry of this process during terminal differentiation of keratinocytes, reducing the efficacy of the barrier against UVB irradiation and water loss (Denecker et al., 2007).

Stratum corneum morphology in Gata3-deficient mice was impaired, with increased numbers of corneocyte layers. It was shown that cystatin A, which is very potent inhibitor of cathepsins, can cause hyperkeratosis when applied topically (Sato et al., 1998). Cystatin upregulation in the K14-Gata3$^{-/-}$ epidermis is likely to cause the enormous thickness of the stratum corneum by inhibiting enzymes, which normally are active in the process of desquamation.

Epithelial tissues provide a barrier between an organism and its environment and perform many additional specialized functions depending on its body site. In order to function correctly, stratified epithelia such as the epidermis have to maintain tight cell-cell adhesion in the living cells and retain the dead, keratinized squames as a protective sheath prior to being sloughed. In the K14-Gata3$^{-/-}$ skin the balance between process of cornification and desquamation is altered. The phenotype of K14-Gata3$^{-/-}$ skin in this study strongly suggests that it is the result of a failure to maintain the balance between proliferation and shedding.

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JEEEEZ... THOSE EMBRYOS ARE LOOKING AT ME!!
..BRRRRR....
LAURA, GATA3 DEFFICIENCY CAUSES OPEN-EYE PHENO-TYPE!
GREAT!!
KASIA! MOJE NOWORODKI MAJĄ OTWARTE OCZY.
OKROPNE STWO ALE GRATULUJĘ!
..MARTINE!
NICE!
...GOSIA!
FAJNIE!
XENIA, DUDI, I HAVE A PHETOTYPE!
?
EKIM, YOU KNOW WHAT?...
NOOKIE? LOOK!
?
NIIIICE!

Chapter 4

Gata3-deficiency leads to eye-open at birth phenotype

Manuscript in preparation

Gata3-deficiency leads to eye-open at birth phenotype

Dorota Kurek, Jacqueline van der Wees, Frank Grosveld

The transcription factor Gata3 is critically involved in many processes during development. Mice lacking Gata3 die in utero in midgestation due to noradrenaline (NA) deficiency of the sympathetic nervous system and can be rescued with NA agonists. Here we show that NA-rescued Gata3 null mutant exhibits an eye-open at birth phenotype. It appears that Gata3 has a dual role in the eyelid closure. Firstly Gata3 controls FGF10 expression in the mesenchyme of the eyelid and secondly withdrawal of Gata3 causes an increase in p57(KIP2) expression and a cessation of proliferation in the eyelid, which inhibits epithelial sheet movement.

Key words: mouse, Gata3, eyelid closure, eye-open at birth phenotype, cell proliferation, p57(KIP2), morphogenetic transition, organogenesis

Introduction

During normal development in mammals, the eyelids grow across the eye, fuse and subsequently reopen. In mice, eyelid formation begins on day 13 of gestation (E13), from E14 to E16 the eyelids grow, flatten across the eye, progressively meet beginning at the inner and outer canthi and fuse tightly with each other (Harris and McLeod, 1982; Li et al., 2001). The eyelashes and the glands lying along the margins of the lids then start to differentiate from this common epithelial lamina before the lids reopen at 14 days after birth (Findlater et al., 1993).

The developing eyelids are composed of loose mesenchyme covered by an epithelial sheet, the epidermis (outer surface) and conjunctiva (inner surface) and the periderm, which covers the epidermis (Weiss and Zelickson, 1975). Only the peridermal and epidermal layers are involved in eyelid fusion; the mesenchymal layers of the upper and lower eyelids remain separate (Pei and Rhodin, 1970). A profusion of rounded periderm cells appears, and they pile up at the leading edges of the advancing eyelids during eyelid growth (Harris and Juriloff, 1986; Harris and McLeod, 1982; Juriloff and Harris, 1989). Once contact is made between the apposed eyelids, these cells flatten and form a strip along the fusion line, until they slough off with the rest of the periderm on day 17 of gestation (Findlater et al., 1993; Harris and Juriloff, 1986; Juriloff and Harris, 1989). When embryonic eyelid development is impaired, mice exhibit an eye-open at birth (EOB) phenotype. Mutations at several distinct loci have been found to cause EOB, often as part of a syndrome with other defects. For example, the EOB phenotype is observed in mice defective in the genes encoding growth factors and their receptors such as activinß1, transforming growth factor α (TGFα), epidermal growth factor receptor (EGFR), fibroblast growth factor receptor 2b (FGFR2b), Heparin-binding EGF-like growth

factor HB-EGF, mitogen activated protein kinase kinase MEKK1, Rho-assciated kinase ROCK, desmocollin 1 (Dsc1) and FGF10 (Chidgey et al., 2001; Juriloff et al., 2005; Li et al., 2001; Luetteke et al., 1993; Mann et al., 1993; Miettinen et al., 1995; Mine et al., 2005; Tao et al., 2005; Threadgill et al., 1995; Thumkeo et al., 2005; Vassalli et al., 1994).
The zinc finger transcription factor Gata3 is essential for proper development of several tissues and organs during embryogenesis and Gata3 mutants die at embryonic day 11 (Pandolfi et al., 1995) due to noradrenaline (NA) deficiency of the sympathetic nervous system and can be rescued to perinatality by feeding heterozygous intercrossed animals with catecholamine intermediates (Lim et al., 2000) or by restoring Gata3 function specifically in SA lineages using human dopamine ß-hydroxylase (hDBH) gene promoter to direct GATA3 transgenic expression (Moriguchi et al., 2006). Here we show that noradrenergic agonists (Pattyn et al., 2000) can rescue midgestational lethality of Gata3 mutants and these foetuses exhibit EOB phenotype.

Materials and methods

Mice

Mice nlsLacZGata3 were described previously (Pata et al., 1999). Noon of the day when the vaginal plug was detected was considered embryonic day 0.5 (E0.5) of development for embryos of the overnight mating.

Rescue of NA deficiency

For rescue experiments, drinking water of pregnant heterozygous Gata3LacZ females was supplemented with 100 µg/ml of L-phenylephrine, 100 µg/ml of isoproterenol, and 2mg/ml of ascorbic acid, from E8.5 onwards.

Histology

Tissues were collected in Tissue-Tec embedding medium (Sakura) and snap-frozen in an ethanol-dry ice bath or fixed in 4% PFA in PBS at 4°C overnight and then subsequently embedded in paraffin and sectioned at 5 µm. 10 µm thick sections were cut in case of freshly frozen tissues, fixed in 4% paraformaldehyde and stained with hematoxylin and eosin.

Immunohistochemistry and X-gal staining

Cryosections were fixed for 10 min in 4% PFA in PBS and washed three times for 5 min in PBS. For FGF10, cyclin A2 and p57(KIP2) immunohistochemistry, samples were subsequently embedded in paraffin and sectioned at 5 µm. After deparaffination sections were boiled in 0.01M citrate buffer (pH 6.0) for 15 min. prior to incubation with primary antibody. As block/diluent was used: 1% BSA, 0.05% Tween in PBS. Primary antibodies that were used at the indicated dilutions were: p63 (1:500 mouse, clone: 4A4, DAKO), cyclin A2 (1:100 rabbit, C-19; sc-596, Santa Cruz) and p57(KIP2) (1:100 goat E-17; sc-1037, Santa Cruz). Appropriate HRP-conjugated goat antibodies (1:100, DAKO) were used to detect primary antibodies. When murine primary antibodies were applied, MOM blocking reagent (Vector Laboratories) was used to block endogenous immunoglobulins. For X-gal

staining sections were fixed 1 min in 0.5% glutaraldehyde, 1% PFA, washed in PBS and incubated in X-gal staining solution for 5 h at room temperature. Sections then were fixed in 4% PFA for 10 min and counterstained with neutral red or hematoxylin. Sections were studied and photographed with an Olympus BX40 light microscope. Sections were studied and photographed with an Olympus BX40 light microscope.

Results

Gata3 is expressed in the developing eyelids

Gata3 expression was shown in the lens fibers and in the periorbital region (Lakshmanan et al., 1999; Oosterwegel et al., 1992). Gata3 expression appears in the periorbital region around embryonic day 11 and later in the developing eyelids is located in the cells adjacent to the conjunctival epithelium and in the epidermis (Fig 1A, B).

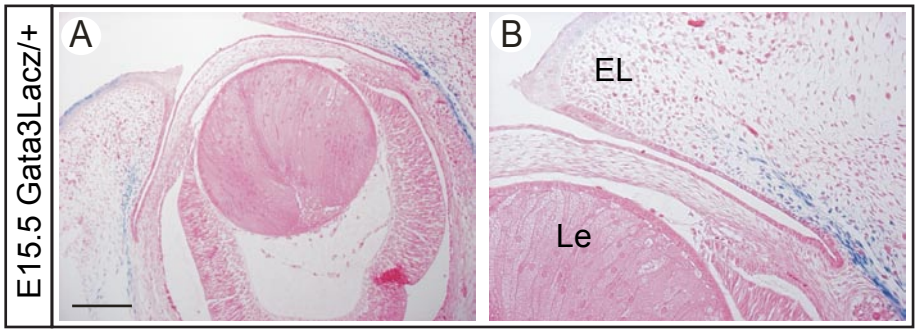

Figure 1. Gata3 expression in the developing eye. X-gal staining of heterozygous Gata3LacZ eyelids at E15.5. LacZ positive cells were located underneath conjunctival epithelium next to the centre and bottom tip of the invaginations and in the epidermis. Scale bar in A 200 μm for A and 100 μm for B.

Midgestational lethality of Gata3 mutants can be rescued by noradrenergic agonists

Gata3 mutants die at embryonic day (E) 11 due to NA deficiency of the sympathetic nervous system (Pandolfi et al., 1995). We have prevented this early death by feeding pregnant mothers with isoproterenol and L-phenylephrine throughout gestation (see Methods). Homozygous mutants were recovered from intercrosses of heterozygotes at E13.5, E15.5 and E16.5 and developing eyelids were compared to control littermates (Fig. 2).

Gata3 is required for eyelid closure

During development of wild-type mice, the eyelids migrate across the surface of the eye and fuse at E16.5. The eyelids are tightly closed at birth and remain fused until their complete separation and reopening around 14 days after birth, a process accelerated by EGF. The eyelids of wild-type embryos were fully open at E13.5 and E15.5 (Fig.2A, C and E) but completely closed at E16.5 (compare Fig 2E,and G).

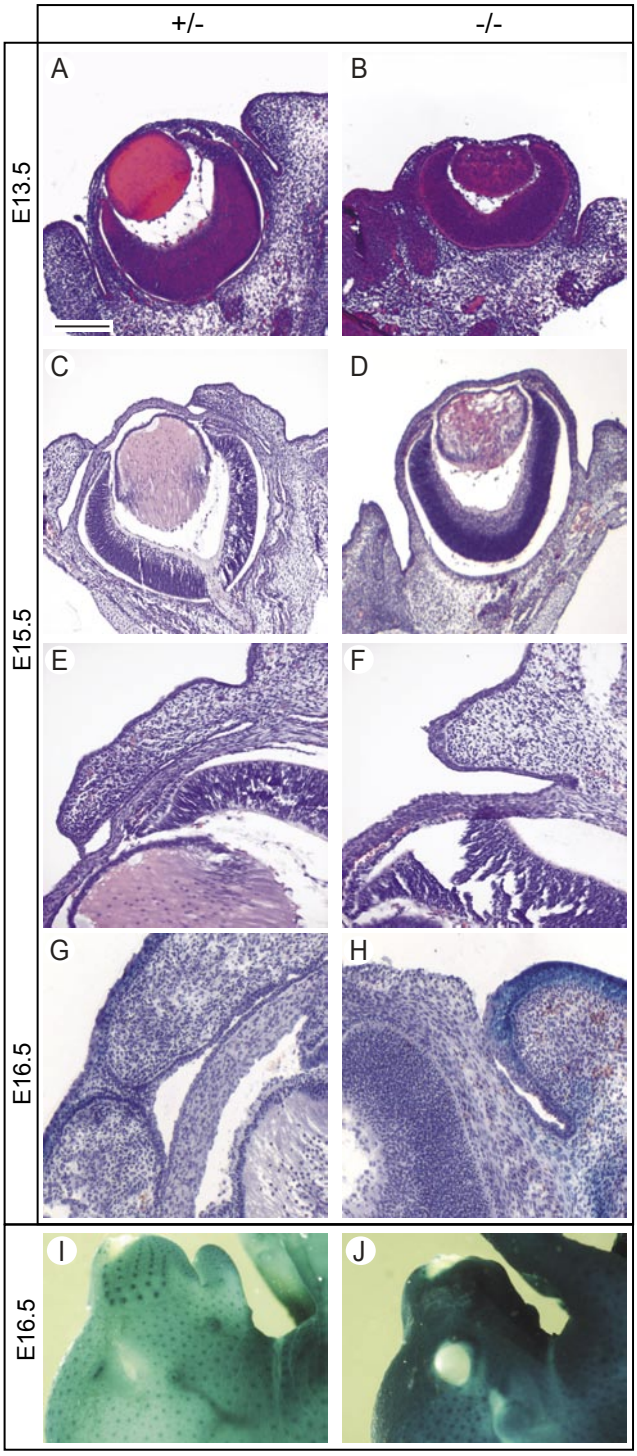

Figure 2. Gata3 is required for the eye closure. A-H: H&E staining the coronal eye sections from control (left panel) and Gata3 null (right panel) fetuses of various gestational ages (E13.5-E16.5) Photograph of the X-gal stained heads of control (I) and Gata3 null (J) at E16.5. Scale bar in A 300 µm for A-D; 150 µm for E-F and 100 µm for G-H.

In contrast, Gata3-null mutant embryos had open eyelids at E13.5, E15.5 and E16.5 (Fig.2B,D,F,H), which remained open until birth. The induction of the eyelid formation does take place in Gata3-null mutants but they fail to move forward. In wild-type mice the protruding epithelial ridge forms the leading edge. In the Gata3-null mutants a round malformed eyelid edge was observed and the leading edge was not formed (Fig.2D)

FGF10 expression is downregulated in Gata3-deficient eyelids

Fgf10-null mutants exhibit eyes open at birth phenotype and it was shown that FGF10 expression can be controlled by Gata3 expression (Lillevali et al., 2006; Tao et al., 2005). To test whether FGF10 signalling is affected in the eyelids of the Gata3 mutants we examined the expression of FGF10 in mutants by immunohistochemistry. This indeed showed that the expression of FGF10 was substantially reduced in E15.5 Gata3 deficient eyelids compare to control littermates Fig. 3 A, B).

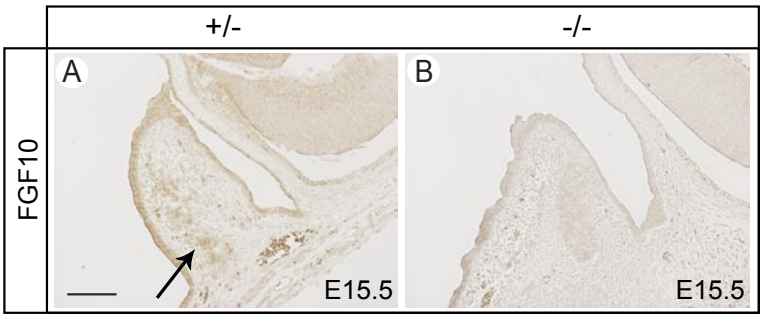

Figure 3. FGF10 expression is downregulated in Gata3-deficient eyelids at E15.5. The arrow points to the expression of FGF10 in mesenchyme. Scale bar in A 150 μm for A-B.

Gata3-deficiency leads to cessation of the cell cycle in the eyelid

Gata3 was implicated in regulation of cell cycle, proliferation and differentiation (Grote et al., 2006; Kouros-Mehr et al., 2006; Kurek et al., 2007). We therefore examined the expression of cell-cycle regulators p63, cyclin A2 and p57(KIP2).

p63 maintains a proliferative potential of basal keratinocytes and promotes stratification of the epidermis (Koster and Roop, 2004). p63 null mutants exhibit EOB phenotype because p63 controls TGFβ expression (Mills et al., 1999) and p63 was shown to control Gata3 expression (Candi et al., 2006; Chikh et al., 2007). p63 is expressed in epidermal nuclei of the developing eyelids and there was no difference observed in the expression levels of p63 between control and Gata3 null mutant (fig.4 A, B).

Cyclin A2, which is expressed in proliferating cells, possesses a unique role in its two-point control of the cell cycle, first by interacting with cdk2 in controlling the G1/S transition into DNA synthesis and then by interacting with cdks 1 and 2

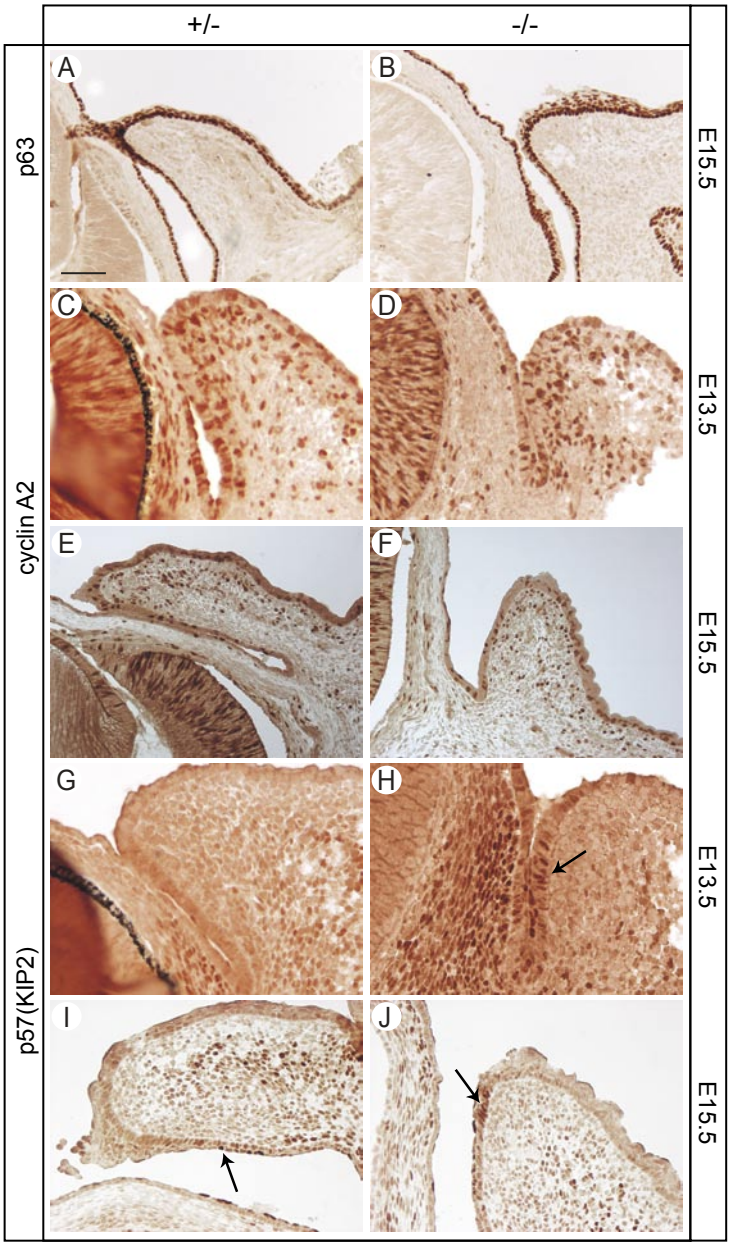

Figure 4. Gata3 has a role in the cell proliferation in eyelid closure. Expression of p63 (A-B), cyclin A2 (C-F) and p57(KIP2) (G-J). The arrows show strong nuclear expression of p57(KIP2). Scale bar in A 150 µm for A-B and E-F; 100 µm for C-D and I-J; 50 µm for G-H.

to control the G2/M entry into mitosis (Elledge et al., 1992; Girard et al., 1991; Kobayashi et al., 1992; Pagano et al., 1992; Rosenblatt et al., 1992). Cyclin A2 positive cells were detected in the mesenchyme and epidermis of the developing eyelids at E13.5 (fig.4C,D) and E15.5 (Fig.4E, F). There was no significant difference between the number of cyclin A2 positive cells in the control and Gata3 mutant eyelids (Fig.4 C-F).

p57(KIP2) negatively regulate progression through the cell cycle by inhibiting a broad spectrum of cyclin–CDK complexes (Sherr and Roberts, 1999). In the wild-type embryos p57(KIP2) expression is absent in the epidermis of the E13.5 eyelid (Fig.4 G) and at E15.5 some p57(KIP2) positive cells appear in the conjunctiva (fig.4 I arrow) while the leading edge of the eyelid is negative. Gata3 mutant eyelids show strong expression of the p57(KIP2) in the epidermis at E13.5 (Fig. 4 H) and E15.5 (Fig.4J) . Interestingly the strongest expression of p57(KIP2) at E15.5 appears in a few cells of the presumptive leading edge of the Gata3 mutant eyelids (Fig.4 J, arrow).

We have looked closely at the expression of p57(KIP2) in the eyelids by comparing them with the expression of the Gata3 driven LacZ transgene (Fig. 5). Expression of LacZ was expanded in the homozygous eyelid which suggests that Gata3 controls its own expression and in the absence of Gata3 protein promoter activity is misregulated.

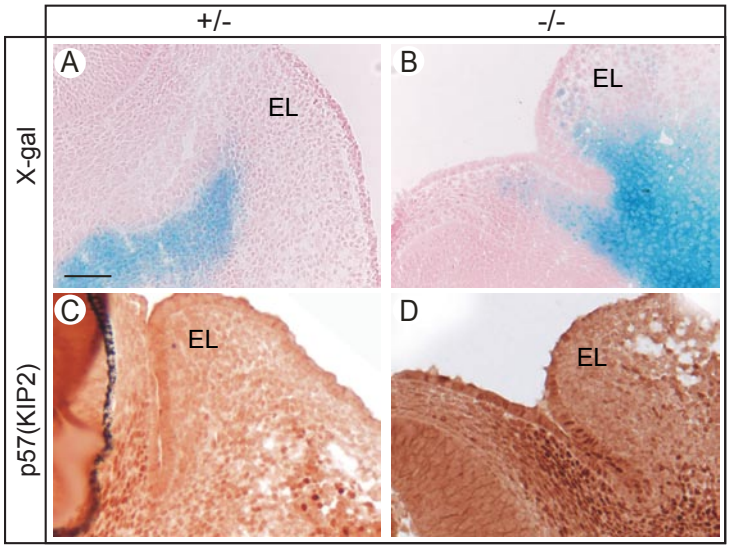

Figure 5. Gata3 deficiency leads to the upregulation of cell cycle inhibitor p57(KIP2) in eyelid closure. A-B; X-gal staining of E13.5 Gata3LacZ heterozygous (A) and homozygous (B) eyelids. C-D: In the control eyelids most of p57(KIP2) cells were located underneath corneal epithelium next to the bottom tip of the invaginations, where Gata3 was not expressed (C). In Gata3 null eyelids, expression of p57(KIP2) was expanded underneath corneal epithelium and it appeared around the bottom tip of the invaginations (D). Scale bar in A 50 µm for A-D. EL: eyelid.

In the heterozygous eyelids, at E13.5, LacZ expression was detected in the mesenchyme of the eyelid, underneath conjunctival epithelium, at the bottom tip of the invaginations but not underneath corneal epithelium (Fig.5 A). In the homozygous eyelids expression of LacZ was expanded in the eyelid, it appeared at both sides of the invagination, underneath corneal and conjunctival epithelium and at the tip of the eyelid underneath the epidermis (Fig.5 B). In the control, at the same stage, most of p57(KIP2) cells were located underneath corneal epithelium next to the bottom tip of the invaginations, where Gata3 was not expressed (Fig.5 C). In the absence of Gata3, expression of p57(KIP2) was expanded underneath corneal epithelium and it appeared around the bottom tip of the invaginations (Fig.5 D). This result suggests that Gata3 controls cessation of the cell cycle in the eyelid. In the presence of the Gata3 in the eyelid, p57(KIP2) expression is not permitted, and the eyelids grow across the cornea. When Gata3 is missing, p57(KIP2) expression is upregulated both in the mesenchyme and epithelium, and the growth is blocked.

Discussion

Gata3 has a dual role in the eyelid closure. On one hand, Gata3 controls FGF10 expression in the mesenchyme of the eyelid. Secondly, withdrawal of the Gata3 causes an increase in p57(KIP2) expression and the cessation of proliferation in the eyelid, which inhibits epithelial sheet movement.

The development of mouse eyelids starts by embryonic day E13 with eyelid folds that extends over the cornea and move towards the centre of the eye: eyelid closure is accomplished at E15.5–E16.5. This process is followed by the fusion of the eyelid epidermis form a closed eyelid that covers the ocular surface and serves as a protective barrier crucial for normal eye development. Although the initial eyelid formation does not require the presence of Gata3 factor, its closure depends on the movements of the epithelial sheets and for that process Gata3 is required.

The fibroblast growth factor (FGF)-FGF receptor (FGFR) signaling network includes more than 20 ligands of the FGF family and four related tyrosine kinase receptors, FGFR1-4 (Itoh and Ornitz, 2004). FGF10 is a secreted molecule from a cell that binds to and activates FGFR2b, an isoform of FGFR2 (Igarashi et al., 1998; Itoh and Ornitz, 2004). The importance of FGF10-FGFR2b signaling during mouse development has been demonstrated by gene targeting studies. As phenotypes found in Fgf10-null or Fgfr2b-null mice are very similar and closely related each other (for example, lack of limbs, lungs, lacrimal glands, and eyelids), although a more severe phenotype is often seen in Fgfr2b-null mice. FGF10 is thought to be a major ligand for FGFR2b during organogenesis requiring epithelial–mesenchymal interactions (De Moerlooze et al., 2000; Makarenkova et al., 2000; Ohuchi et al., 2000; Sekine et al., 1999). It is known that Gata3 is an important regulator of Fgf10 expression in otic epithelium. Its inactivation leads to a loss of Fgf10 expression in otic epithelium and auditory ganglion demonstrating that Gata3 is an important

regulator of Fgf signaling during otic development. The lack of Fgf10 expression in Gata3 deficient otic epithelium and activation of Fgf10 expression by Gata3 in cell culture suggest that Gata3 could be the first factor to control Fgf10 expression in the inner ear (Lillevali et al., 2006). Here we show that Gata3 deficiency in the epithelial sheet causes downregulation of FGF10 expression during eyelid development. FGF10 is dually required for proliferation and coordinated migration of epithelial cells during mouse eyelid development by reorganization of the cytoskeleton, through the regulation of activin, TGFα and SHH signalling (Tao et al., 2005). Loss of Fgf10 expression causes EOB. However, the molecular and cellular events occurring during eyelid development and the interactions among the signalling molecules have not been fully elucidated.

Members of the Cip/Kip family of cyclin-dependent kinases inhibitors (CKIs) are well characterized for their role as negative regulators of G_1-phase cell-cycle progression (Sherr and Roberts, 1999). In eukaryotic cells, progression through the cell cycle is governed by a suite of cyclins and cyclin-dependent kinase (CDKs) complexes (Murray, 2004). Regulation of cyclin–CDKs complexes occurs at multiple levels, including assembly of cyclin and CDK subunits, inhibitory and activating phosphorylation and dephosphorylation events, and association of cyclin–CDK complexes with CKIs. During these regulatory processes, cyclin–CDK complexes positively drive progression of the cell cycle, whereas by binding to and inactivating cyclin–CDKs, CKIs negatively regulate progression through the cell cycle. Based on their sequence homology and specificity of action, CKIs are divided into two distinct families: INK4 and Cip/Kip (Sherr and Roberts, 1999). Members of the INK4 family, namely p15, p16, p18, and p19 specifically inhibit the activity of CDK4 and CDK6, whereas Cip/Kip members, that is, p21, p27, and p57 inhibit a broader spectrum of cyclin–CDK complexes (el-Deiry et al., 1993; Gu et al., 1993; Harper et al., 1993; Lee, M. H. et al., 1995; Polyak et al., 1994; Toyoshima and Hunter, 1994).

Until recently, Cip/Kip members were almost solely viewed as nuclear proteins with a principal function of inhibiting cyclin–CDK activity and hence, cell-cycle progression. However, emerging studies now suggest that Cip/Kip proteins play additional roles outside of the nucleus (Coqueret, 2003). Several papers have shown that p57(KIP2) modulates subcellular localization of LIMK, a serine/threonine kinase involved in the regulation of actin filaments (Lee, S. and Helfman, 2004; Tanaka et al., 2004; Yokoo et al., 2003). Moreover it was shown that p57(KIP2) acts as an endogenous inhibitor of JNK and JNK-deficient mice exhibit an eyelid closure defect. Upregulation of p57(KIP2) in the tip of the leading edge of the developing eyelid inhibits proliferation of epithelial cells and by JNK inactivation inhibits coordinated migration of epithelial cells. Our results strongly suggest that Gata3 is a direct regulator of these pathways.

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THESIS
FINALLY..
DONE!!!

Chapter 5

Discussion

Summary and discussion

Gata3, like other GATA proteins binds to its cognate DNA binding sites and interacts with other proteins. Whether Gata3 is activating or inhibiting the expression of a gene is dependent on the co-factors it binds, the protein content of the cell, the levels of Gata3 expression, the cell cycle stage or signals from the environment. In case of the skin and HF the direct targets of Gata3 are as yet not known. From the studies shown in this thesis it is clear that Gata3 has a different role in epidermal cells and HF cells. The expression levels of Gata3 change during HF cycle; from a high level of expression in the anagen to very limited or no expression in the telogen. The level of expression is critical since in the Gata3-knockdown, with Gata3 levels less than 50%, the phenotype of the mouse varies between littermates and change in the same mutant during life, e.g. they suffer from cyclic alopecia or the hair phenotype varies from normal to severely disturbed (van der Wees and Kurek, unpublished observation). Gata3 also appears to have an important role in the human skin disorders; the expression of Gata3 is altered in the lesions of vitiligo patients (Eerik Aunin, unpublished observation) and the expression levels change dramatically upon UV treatment of psoriatic lesions (Leslie van der Fits, Emoke Racz, unpublished observation).

Summary of the thesis

In Chapter 1 the role of Gata3 was investigated in epidermis and hair follicle development by generating an epidermis-specific Gata3$^{-/-}$ mouse model. Deletion of the Gata3 gene in the murine epidermis and hair follicles results in postnatal growth and developmental abnormalities along with aberrant hair growth and cycle, abnormal hair follicle organization and pigmentation. After the first hair cycle new visible hairs were not produced. A laser-capture approach was used to microdissect the proximal end of hair follicles and their transcriptome profiles were analysed using an Affymetrix platform. This approach revealed several processes pertinent to the cell cycle, epithelial growth and differentiation and signal transduction pathways were affected. Importantly, we identified a broad, uniform decrease in the expression of most genes associated with the transition of the mitotic cell cycle and an increase of genes encoding keratins and keratin associated proteins. The lack of hair follicle regeneration and increased thickness of epidermis suggested that Gata3 is a crucial component of the choice between forming different layers of the hair follicle versus basal epidermal cells. If Gata3 levels are greatly diminished, matrix cells do not develop into functional IRS cells and basal epidermis is oversupplied with cells, which express proliferation genes and bulge specific marker genes.

Chapter 2 decribes the analysis of the protein composition of the hairs, their follicles and surrounding squames of the K14-Gata3$^{-/-}$ mice to gain insight into the molecular mechanism of the thickened epidermis. Proteomic profiling and immunohistochemistry revealed a change in expression of epidermal differentiation

markers and that the phenotype of Gata3 deficient skin is the result of a failure to maintain the balance between proliferation and shedding. In vitro differentiation of primary keratinocytes showed that K14-Gata3$^{-/-}$ keratinocytes appear to remain longer in the early differentiation state when compared to control cells, which would explain the increased thickness of stratified and cornified layers in the K14-Gata3$^{-/-}$ mice. Chapter 2 demonstrates that Gata3 is important for the balance between the process of cornification and desquamation in the skin.
The results presented in chapter 3 suggest that Gata3 controls the cessation of the cell cycle in the developing eyelid. In the presence of the Gata3 in the eyelid, p57(KIP2) is not expressed and the eyelids grow across the cornea. Absence of Gata3 causes an increase in p57(KIP2) expression. The rise in the level of this inhibitor of cell cycle progression causes a cessation of proliferation in the eyelid, which results in the inhibition of the movement of the epithelial sheet and consequent growth of the eyelid.

Cell fate specification

The six vertebrate factors (Gata1-6) are important in cell-fate specification during development (reviewed in(Patient and McGhee, 2002); e.g., Gata4, was shown to have a role in cell fate specification. It is responsible for the maintenance of regional identities along the gastrointestinal tract and is a major and essential positional signal required for the maintenance of differential functions between jejunum and ileum in vivo. When Gata4 is conditionally deleted from the villi enterocytes, the jejunum lacks its identity and starts to express illeal markers (Bosse et al., 2006).
It was shown before that Gata3 is an important factor for cell fate decisions in T cell development. Expression of Gata3 and repression of the T-box transcription factor T-bet are required for Th2 cell commitment. Ectopic expression of Gata-3 in T cells induces Th2-specific cytokine expression not only in developing Th1 cells, but also in otherwise irreversibly committed Th1 cells and Th1 clones (Lee et al., 2000). GATA-3 plays a role in pathfinding of a population of neurons in rhombomere 4 that extend their axons across the floor plate of rhombomere 4. Both GATA-3 heterozygous and GATA-3 null mutant mice show unusual axonal projections, such as misrouted crossing fibers and fibers in the facial nerve, that are absent in wild-type littermates. This suggests that GATA-3 is involved in the pathfinding of efferent neuron axons that navigate to the ear (Karis et al., 2001). Gata-3 is also a critical regulator of luminal differentiation and its deficiency leads to an expansion of luminal progenitors and a concomitant block in differentiation during mammary gland development (Asselin-Labat et al., 2007; Kouros-Mehr et al., 2006).
We have proposed that Gata3 is a crucial component of the choice between forming different layers of the hair follicle versus basal epidermal cells. Within the epidermal layer it is important for the balance between the process of cornification and desquamation.

Signaling pathways

Little is known about the pathways that regulate Gata3 activity. We have proposed that Gata3 is involved in some signaling pathways in the skin e.g. the Notch, BMP and Wnt signaling pathways. Recently it was reported that GATA-3 is activated in response to Notch-Delta signaling as an early and essential event in T lineage specification (Amsen et al., 2007; Fang et al., 2007). BMPs regulate Gata3 (Andl et al., 2004; Kobielak et al., 2003) and vice versa (Kurek et al., 2007) to maintain appropriate Gata3 expression levels.

GATA-3 also appears to integrate information from extracellular TGF-β signals by physically and functionally interacting with the signal-activated factor Smad3. The interaction between GATA-3 and Smad3 may allow TGF-β signaling to access the promoters of specific sets of genes involved in cell fate and differentiation in the immune and nervous systems (Blokzijl et al., 2002).

Gata and cell cycle

Gata3 involvement in the cell cycle was already implicated before, but the molecular mechanisms of its action remain unresolved.

Upon the deletion of Gata3 specifically in the skin, genes associated with the cell cycle machinery are downregulated in the HF but upregulated in the epidermis (Kurek et al., 2007).

The eye-open at birth studies show that Gata3 downregulation allows expression of cell cycle inhibitor p57kip2 in the mesenchyme of the developing eyelid and in the epidermis of the eyelid leading edge. In addition, Gata3 downregulation in the lens alters the differentiation of the fiber cells; the equatorial zone where the proliferating epithelial cells give rise to fiber cells is not very well defined i.e. cell cycle inhibitors are downregulated and proliferating cells are still present among fiber cells (Kurek, unpublished observation).

In the embryo, the inactivation of Gata3 causes an uncontrolled cellular proliferation in the nephric duct of the embryonic kidney (Grote et al., 2006) and the acute loss of Gata3 in adult mammary glands leads to widespread cellular proliferation suggesting that Gata3 plays a direct role in maintaining the quiescent state of differentiated luminal cells (Kouros-Mehr et al., 2006).

From all these examples it is clear that Gata3 has a role in the regulation of cell-cycle. And this role is dependent on the cell identity i.e. the removal of Gata3 expression in the skin has an opposite effect on HF versus epidermis. However, the mechanism of action of Gata3 during the cell cycle remains unknown.

Other members of GATA family have also been shown to be involved in cell cycle regulation. Gata2 expression, in leukemic and normal hematopoietic cells, oscillates during the cell cycle, such that expression is high in S phase but low in G_1/S and M phase (Koga et al., 2007). Gata2 has the ability to inhibit the proliferation of neural progenitors and this correlates with the control of cyclin D1 transcription and of the expression of the p27/Kip1 protein (El Wakil et al., 2006).

A variety of GATA-1 target genes have been previously identified, that are involved in cell cycle regulation or have known functions in proliferation and differentiation processes such as Bcl-2, c-myc and c-myb. Our lab has previously shown that GATA-1 together with Gfi1b binds to the c-myb promoter in induced MEL cells, suggesting a role of this complex in proliferation arrest that accompanies terminal erythroid differentiation (Rodriguez et al., 2005). Gata1–deficient megakaryocytes express less cyclin D1 which is a direct GATA-1 target gene and increased levels of p16 compared to control (Muntean et al., 2007). Furthermore, Gata1 binds to the retinoblastoma (pRb) protein, which is a regulator of cell cycle progression (Whyatt et al., 1997).
Gata6 induces p21(Cip1) expression, downregulation of cyclin A and G1 cell cycle arrest (Nagata et al., 2000; Perlman et al., 1998).
Moreover, in the plant Arabidopsis thaliana, GATA-3–like transcription factor HAN is important for controlling cell proliferation and differentiation. Ectopic HAN expression causes growth retardation, aberrant cell division patterns, and loss of meristem activity and in the event of HAN loss of function, the meristematic structure becomes disorganized, leading to meristem size reduction, reduced organ numbers and size, and organ fusion (Zhao et al., 2004).

Future directions

We will further address the role of Gata3 in the postnatal hair follicle development using the K14 inducible cre system. Provided sufficient material can be obtained the use of ChIP/chip or ChIP/seq techniques would be obvious methods to identify which of the genes from the microarray analysis list, are direct Gata3 targets. This would help to build the pathways in which Gata3 is involved during the morphogenesis of epidermis and HF.
Verri et al. showed in one case of HDR patient bilateral complete cataract, which suggests that Gata3 deficiency, leads to cataracts in human (Verri at al 2004). The role of Gata3 in the lens should be addressed. The ocular lens provides an ideal in vivo model to signaling mechanisms for tissue induction, cell fate determination and pattern formation (Coulombre and Coulombre, 1963, Grainger, 1992 and Spemann, 1901). The highly ordered lens architecture and differentiation pattern is altered in the Gata3 mutant and should be studied in the greater detail. Also using a lens specific Le-cre transgenic mouse together with Gata3 floxed will allow looking at lens development postnatally (Ashery-Padan et al., 2000).
Although the initial eyelid formation in the Gata3 mutant is not inhibited they fail to grow across the eye and fuse during the fetal stage in mice, which leads to an eye-open at birth (EOB) phenotype. To continue the studies on the eyelids closure of those animals we aim to examine whether exogenous FGF10 protein can rescue EOB phenotype of Gata3 mutants in organ culture of the eyelid anlagen.

We will examine also in greater detail the other members of activin and TGFα-EGFR signaling. In addition to that the role of Gata3 in cell cycle regulation will be studied by using e.g. ChIP. The p57(KIP2) promoter has putative GATA binding sites and it is important to show whether Gata3 interacts with those sites and if yes, what other proteins, together with Gata3, are involved in inhibiting p57(KIP2) expression. From studies with Gata1 the obvious candidates would be Gfi-1b or FOG2 interacting with the NuRD complex (Rodriguez et al., 2005).

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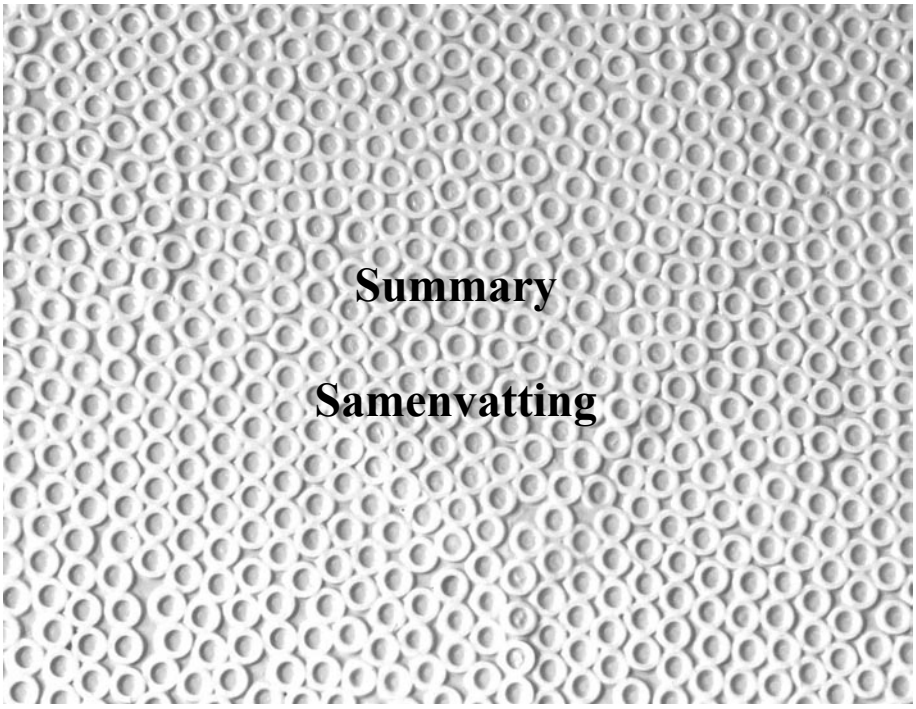
Summary
Samenvatting

Summary

The transcription factor Gata3 is critically involved in many developmental processes. It is essential for proper development of the largest organ of our body – the skin. Gata3 expression appears in the epidermis during embryonic stages and is maintained throughout life. Within hair follicles, its expression changes with the cycle of hair growth. Using a conditional approach that allows the inactivation of the Gata3 upon the activation of Cre recombinase, I ablated Gata3 expression specifically in the epidermis and hair follicles of an animal model. Deletion of the Gata3 gene in the murine epidermis and hair follicles results in aberrant hair growth and cycle. It causes hyperproliferation of the epidermis and increased thickness of the outermost layer of the skin; stratum corneum. Hair development is delayed in these mice and hairs that do grow are short and thick. After the first hair cycle, new visible hairs are not produced resulting in bald mice.

Analysis of gene expression in the hair follicles has shown that a reduction in Gata3 expression levels alters many processes and signaling pathways e.g. cell cycle or epithelial growth and differentiation. The lack of hair follicle regeneration and increased thickness of the epidermis suggests that Gata3 is crucially involved in the decision between forming different layers of the hair follicle versus basal epidermal cell renewal. If Gata3 levels are greatly diminished, the epidermal cells proliferate faster and undergo premature differentiation. In addition, the balance between the processes of cornification and desquamation is altered. As a result, the skin of mice lacking Gata3 is covered with an increased amount of squames.

Gata3 null mutation in mice leads to embryonic lethality due to noradrenaline deficiency of the sympathetic nervous system. Administration of noradrenergic agonists to pregnant dams prolongs the life span of knockout fetuses up to birth. These rescued Gata3 null mutants exhibit an eye-open at birth phenotype. Withdrawal of Gata3 causes an increase in expression of cell cycle inhibitor p57(KIP2) and inhibition of growth of the eyelid.

These findings point towards a role of Gata3 in the regulation of cell cycle, but its mechanism of action is still being elucidated.

Samenvatting

Transcriptiefactor Gata3 speelt een belangrijke rol bij een groot aantal embryonale ontwikkelingsprocessen. Gata3 is van essentieel belang voor de correcte ontwikkeling van het grootste orgaan van ons lichaam - de huid. Gata3 komt tot expressie in de epidermis (opperhuid) tijdens embryonale stadia en ook gedurende het hele verdere leven. Binnenin de haarfollikels varieert de expressie van Gata3 mee met de haargroeicyclus. Met behulp van een muizenmodel waarin de expressie van Gata3 specifiek in bepaalde celtypen kan worden onderdrukt (door het aanschakelen van een eiwit genaamd Cre-recombinase) heb ik de expressie van Gata3 specifiek in de epidermis en haarfollikels uitgezet. Uitschakeling van Gata3 in deze weefsels resulteerde in afwijkingen in de haargroei en in een verstoorde haarcyclus. Het effect was hyperproliferatie van de epidermis en een verhoogde dikte van de buitenste laag van de huid (stratum corneum). Haarontwikkeling is vertraagd in deze muizen en de haren die groeien zijn kort en dik. Na de eerste haarcyclus worden geen nieuwe zichtbare haren geproduceerd, met als resultaat kale muizen.

Analyse van de expressie van andere genen in de mutante haarfollikels heeft aangetoond dat een vermindering van het expressieniveau van Gata3 invloed heeft op vele processen en signaaltransductienetwerken zoals de celcyclus en de epitheliale groei en differentiatie. Het gebrek aan regeneratie van haarfollikels en de toegenomen dikte van de epidermis geeft aan dat Gata3 cruciaal is in de keuze tussen het vormen van verschillende lagen van de haarfollikel tegenover basale epidermale celvernieuwing. Als het niveau van Gata3 sterk verminderd is delen de epidermale cellen sneller en ondergaan ze voortijdige differentiatie. Bovendien verandert het evenwicht tussen de processen van verhoorning en afschilfering. Daardoor is Gata3-loze muizenhuid overdekt met een verhoogde hoeveelheid schilfers.

Complete uitschakeling van Gata3 in muizen resulteert in embryonale dood, veroorzaakt door noradrenalinedeficiëntie van het sympathische zenuwstelsel. Het toedienenen van noradrenerge agonisten aan zwangere vrouwtjesmuizen verlengt de levensduur van de mutante foetussen tot aan de geboorte. Deze “geredde” Gata3 mutanten hebben een open-ogen-bij-geboorte fenotype. Afname van Gata3 veroorzaakt een verhoging van expressie van de celcyclusremmer p57(KIP2) en remming van de groei van het ooglid.

Deze bevindingen wijzen op een rol van Gata3 in celcyclusregulatie, maar het precieze werkingsmechanisme moet nog steeds worden opgehelderd.

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May 2007 3rd Annual Meeting Network of Excellence “Cells Into Organs”, Les Embiez, France

List of Publications

Dorota Kurek, George A. Garinis, J. Hikke van Doorninck, Jacqueline van der Wees and Frank G. Grosveld, **Transcriptome and phenotypic analysis reveals Gata3-dependent signalling pathways in murine hair follicles.** *Development* 134, 261-272 (2007)

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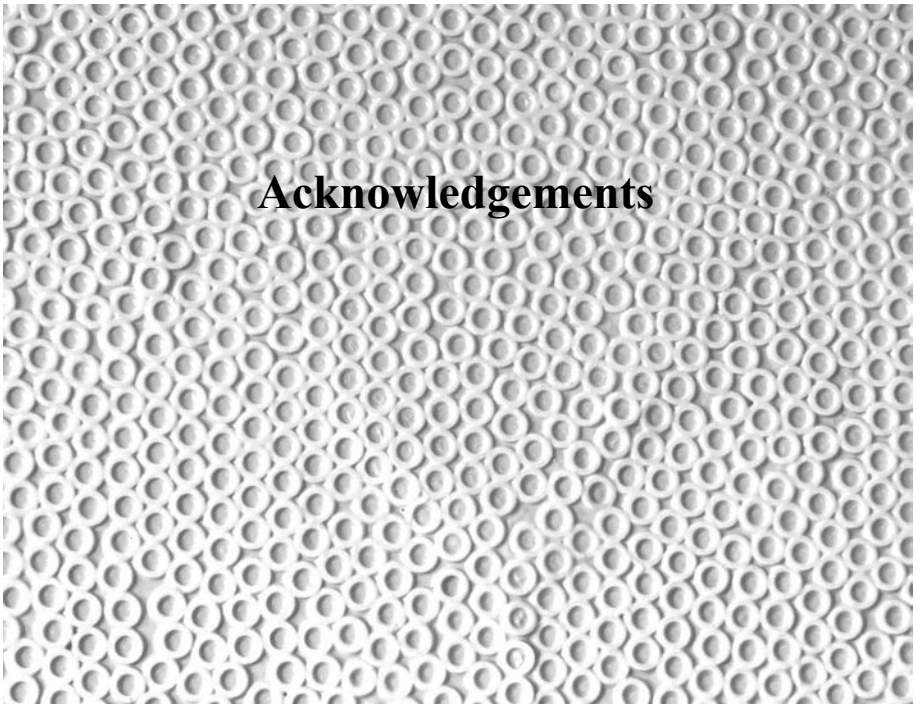
Abbreviations

APC	*antigen-presenting cell*
BMP	*bone morphogenic protein*
BrdU	*5'-bromo 2'-deoxy-uridine*
CD34	*cluster of differentiation 34*
CDK	*cyclin-dependent kinase*
ChIP	*Chromatin immuno-precipitation*
DBH	*dopamine b-hydroxylase*
DN	*double negative*
DNA	*deoxyribonucleic acid*
DP	*dermal papilla*
E10.5	*embryonic day 10.5*
EGFR	*epidermal growth factor receptor*
EOB	*eye-open at birth*
EPUs	*epidermal proliferative units*
ER	*estrogen receptor*
FACS	*fluorescent activated cell sorting*
FGF	*fibroblast growth factor*
FGFR2b	*fibroblast growth factor receptor 2b*
H&E	*hematoxiline and eosine*
HB-EGF	*heparin-binding EGF-like growth factor*
HDR	*hypoparathyroidism, sensorineural deafness, and renal insufficiency*
HR	*hair follicle*
HRP	*horse radish peroxidase*
IFN- γ	*interferon-γ*
Ig	*immunoglobulin*
IHCs	*inner hair cells*
IL-2	*interleukin 2*
IRS	*inner root sheath*
Krtap	*keratin-associated protein*
MEKK1	*mitogen activated protein kinase kinase*
NA	*noradrenaline*
OHCs	*outer hair cells*
ORS	*outer root sheath*
P7	*postnatal day 7*
PBS	*phosphate buffered saline*
PFA	*paraformaldehyde*
PTH	*parathyroid hormone*
r4	*rhombomere 4*
RNA	*ribonucleic acid*
TEBs	*terminal end buds*
TGFα	*transforming growth factor α*
Th1	*T helper type 1*



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