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

Dorota Kurek¹, George A. Garinis², J. Hikke van Doorninck³, Jacqueline van der Wees¹ and Frank G. Grosveld¹,*

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 signalling 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.

KEY WORDS: Gata3, Hair follicle, Epidermis, Laser capture microscopy, Microarray, Mouse

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 that undergo 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 that 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).

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
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).

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 loss of abdominal hair (P19, Fig. 1I,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).

Aberrant hair follicle organization and abnormal hair pigmentation in K14-Gata3–/– mice
At P0, K14-Gata3–/– HFbs appeared normal. However, beginning at P3, K14-Gata3–/– HFbs 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 HFbs 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). Subsequently, the K14-Gata3–/– HF entered telogen after a delayed catagen lasting at least 6 days instead of the normal 3 days (see Fig. 2F). Subsequently, the K14-Gata3–/– HF entered telogen after a delayed catagen lasting at least 6 days instead of the normal 3 days (see Fig. S1A-F in the supplementary material) and the HF proximal end failed to reform (see Fig. S1I,J in the supplementary material). New visible hairs were not produced, although some developing HFbs and small cysts were visible (see Fig. S1J in the supplementary material).

The newly formed HFbs 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 al., 2003). Evaluation of Gata3 expression in Gata3 LacZ knock-in mice and by in situ hybridization revealed Gata3 to be readily expressed in other skin structures (Pata et al., 1999) (Fig. 3 and see Fig. S2 in the supplementary material). 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 see Fig. S2 in the supplementary material) and in certain germinative layer cells surrounding the dermal papilla (Fig. 3F,J,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. 2T). K14-Gata3–/– skin sections, at different ages, revealed gradual, pronounced epidermal thickening including that of basal and suprabasal layers (see Fig. S3B,D in the supplementary material) as well as the development of hyperkeratosis (increased thickness of stratum corneum; Fig. 2C-H and see Fig. S3B,C in the supplementary material). 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 obtain 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; Fig. 4H and see Table S1 in the supplementary material). 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 (Fig. 4H, and see Table S1 in the supplementary material) led us to identify:

1) The downregulation of genes associated with the mitotic cell cycle and replication, and the upregulation of genes associated with nucleosomal assembly, suggesting that cells in the K14-Gata3–/– HF require Gata3 to progress successfully through the cell cycle.

2) The downregulation of pro-apoptotic genes and the upregulation of anti-apoptotic genes. This supports 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). This is likely to reflect the presence of incompletely differentiated cell types.

4) The suppression of all genes associated with immune, inflammatory and defense responses and the downregulation of genes associated with fatty acid synthesis and storage, confirming the considerable loss of fat in the dermis of K14-Gata3–/– mice (Fig. 2H).

5) The up- or downregulation of genes associated with the FGFR, Notch, Wnt and BMP signaling pathways. PCR evaluation of the expression levels of several genes in the HF of K14-Gata3–/– mice and wt littermates confirmed the validity of the microarray data (see Fig. S7 in the supplementary material).
**Fig. 4. Transcriptional 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) 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 transcriptional 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-tailed analysis of variance.
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 the 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 is likely to contribute 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 are 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 analyses 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 increased cyclin D1 expression in the ORS (see Fig. S4 in the supplementary material).

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 Krtap16 gene family, ranging from ~6- to 33-fold, which was further

Fig. 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.
confirmed by in situ mRNA evaluation of Krtap16-7 (Fig. 6A,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) (Fig. 6C), whereas K14-Gata3–/– 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–/– 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. 

AE15, an antibody that recognizes trychohyalin found in characteristic granules of the IRS and medulla (O’Guin et al., 1992) (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).

Consistent with the absence of Huxley’s layer and the cuticle of the IRS in K14-Gata3–/– HFs, expression of μ-crystallin (Crym – Mouse Genome Informatics), known to co-localize with Gata3 in the IRS (Fig. 6G) (Aoki et al., 2000), was diminished in K14-Gata3–/– HFs (Fig. 4H and Fig. 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 young (P1, Fig. 7A,B) and mature (5 months, Fig. 7C,D) skin. In P4 skin, K10 expression was not different between the wt and K14-Gata3–/– mice (Fig. 7E,F). 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 colocalized (Fig. 7K). At later stages, the number of K6-expressing cells in the skin increased substantially (Fig. 7J-L). 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 findings (Fig. 4H). Interestingly, the basal epidermal layer and ORS were almost twice as thick in K14-Gata3–/– compared with 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 of the prominent signal transduction pathways known to be involved in HF morphogenesis were significantly over-represented in the K14-Gata3–/– HF transcriptome, including the Wnt and BMP families, sonic hedgehog and Notch (Fig. 4G-H). Wnt3, Wnt10b, and Wnt11 were upregulated in K14-Gata3–/– HFs, whereas Wnt5a and Wnt6 were downregulated compared with controls (Fig. 4H). 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 (Fig. 4H and Fig. 8B), a finding 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) (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 (Fzrb, an inhibitor of Wnt signaling) was significantly downregulated (Fig. 4H). In agreement, we detected strong nuclear β-catenin staining in K14-Gata3–/– HFs (Fig. 8E,F). Shh along with Gli1, which is a known target and transducer of Shh signaling, the hedgehog-interacting protein (Hhip) and patched homolog 2 (Pch2) 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. In situ mRNA hybridization showed Notch1 to be expressed in precursor cells of cortex, cuticle, and IRS (Fig. 8I) (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 role in Shh and Notch signaling pathways, though this effect is likely to be 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

---

Fig. 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-labeling 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.

Fig. 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 β-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 Gl1 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.
DEVELOPMENT

S100A6 were dramatically upregulated in the distal K14 (Blanpain et al., 2004; Kizawa and Ito, 2006). Compared to wt skin, keratin 15 and S100A6 (Blanpain et al., 2004; Kizawa and Ito, 2006) of the bulge, we investigated the expression of stem cell markers (Fig. 9B).

The majority of transcription factors are upregulated in K14-Gata3+/− HFs
Several transcription factors demonstrated deviant expression profiles in the K14-Gata3+/− HF (see Table S1 in the supplementary material), 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 (Zfp1m – Mouse Genome Informatics) to form repressive chromatin complexes (Rodriguez et al., 2005). Cutl1, which 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 are likely to 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; 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 (see Fig. S6 in the supplementary material), 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. Interestingly, K6, the marker for proliferating cells, is absent in epidermis from both the inside and outside of the ear, but is present in HF (Fig. 9A). In K14-Gata3+/− the epidermis on the outside of the ear expresses K6, whereas the inside, that never had HFs, remains K6-negative (Fig. 9B).

DISCUSSION
Gata3 is essential for proper maintenance of the epidermis and hair follicle cycling
Deletion of the 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, and 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. We implemented a full mouse transcriptome analysis in laser-captured HFs to get an unbiased insight into: (1) the implicated biological processes; (2) the signalling mechanisms involved; and (3) 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 proliferation and cell fate decisions in the matrix, which is likely to

![Fig. 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.](image-url)
shift 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, which is likely to reflect the delayed onset of growth and differentiation. In agreement, several pro-apoptotic genes were downregulated whereas 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 losing 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. Although 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 Lef1, a downstream Wnt mediator, demonstrate a reduced number of body hairs (van Genderen et al., 1994), whereas ectopic expression of either Lef1 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 (Fig. 4, Fig. 8E,F), which probably underlies the abnormal hair formation (DasGupta and Fuchs, 1999). Our results are in agreement with those obtained in Bmp6^{Δ/−}, mice, which have decreased levels of Gata3 (Andl et al., 2004; Ming Kwan, 2004; Yuhki et al., 2004).

Interestingly, all BMP family genes whose expression changes significantly in the K14-Gata3^{−/−} mice (Bmp6, Bmp8a and Bmp15) were upregulated (Fig. 5H). Moderately elevated Bmp6 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 Bmp6 in the 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 (see Table S1 in the supplementary material) only Fgfr5 was downregulated in K14-Gata3^{−/−} mice, whereas Fgfr2 demonstrated increased mRNA expression levels. Mutation of Fgfr5 (Hebert et al., 1994) or expression of a dominant negative Fgfr2 (Schlake, 2005) leads to the growth of long thin hair, whereas Fgfr2^{Δ/−} mice (Petiot et al., 2003) have a 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, Fgfr5 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 directly affecting 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, including Hoxc13 (Godwin and Capeccci, 1998; Tkatchenko et al., 2001), BMP genes (Blessing et al., 1996; Botchkarev et al., 2002) and 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 modulator 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 negatively regulated target of Gata3. In turn, BMPs regulate Gata3 (Andl et al., 2004; Kobielał 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 signalling pathways.

We are grateful to Jos Jonkers for the K14 cre mice; Wilfred van Úcken and his staff of the Center of Biomics for running the microarrays; Heleen van Beusekom for help with SEM; Leslie van der Fits for inflammation studies; Riccardo Fodde and Patrick Franken for anti-keratin 6 and 14 Abs; T. T. Sun (NY
Development

References


