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Abnormal epidermal differentiation and impaired epithelial-mesenchymal tissue interactions in mice lacking the retinoblastoma relatives p107 and p130

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SUMMARY

The functions of p107 and p130, members of the retinoblastoma family, include the control of cell cycle progression and differentiation in several tissues. Our previous studies suggested a role for p107 and p130 in keratinocyte differentiation in vitro. We now extend these data using knockout animal models. We found impaired terminal differentiation in the interfollicular keratinocytes of p107/p130-double-null mice epidermis. In addition, we observed a decreased number of hair follicles and a clear developmental delay in hair, whiskers and tooth germs. Skin grafts of p107/p130-deficient epidermis onto *NOD/scid* mice showed altered differentiation and hyperproliferation of the interfollicular keratinocytes, thus demonstrating that the absence of p107 and p130 results in the deficient control of differentiation in keratinocytes in a cell-autonomous manner. Besides normal hair formation, follicular cysts, misoriented and dysplastic follicles, together with aberrant hair cycling, were also observed in the p107/p130 skin transplants. Finally, the hair abnormalities in p107/p130-null skin were associated with altered Bmp4-dependent signaling including decreased ΔNp63 expression. These results indicate an essential role for p107 and p130 in the epithelial-mesenchimal interactions.

Key words: Mouse, Keratinocytes, p107, p130

INTRODUCTION

The skin plays an essential role as a protective barrier between the organism and the environment. This function is exerted by the epidermis and stems from a finely regulated differentiation program that takes place progressively as committed cells in the basal compartment move upwards to the epidermal surface. This process is characterized by the sequential expression of different proteins, coincident with the phenotypic evolution from basal cell to the mature, nonviable squame. Proliferative basal cells express the keratin pair K5+K14 switching to the pair K1+K10 expression when differentiation and migration begin. Finally, loricrin and filaggrin are expressed during the latest differentiation stages (reviewed by Byrne, 1997; Byrne et al., 1994; Fuchs, 1998; Fuchs and Byrne, 1994). Taken together, the epidermis represents a perfectly suited model for studying proliferation and differentiation. However, the molecular mechanisms governing epidermal differentiation are still largely unknown.

The skin is also characterized by the presence of epidermal appendages. Among them, the hair follicles are of a particular interest. These structures develop through a series of epithelium-mesenchyme interactions (reviewed by Fuchs, 1998; Fuchs et al., 2001; Fuchs and Raghavan, 2002; Hardy, 1992; Millar, 2002), which are similar to those regulating the morphogenesis of other ectodermal organs such as tooth (reviewed by Jernvall and Thesleff, 2000). Among the different signals involved in hair morphogenesis, recent experimental evidences have demonstrated that several components of the Wnt pathway are essential. In fact, the downstream effectors of Wnt signaling, β-catenin and Lef1, are expressed in epithelial and mesenchymal compartments as soon as hair follicle formation is induced (Huelsken et al., 2001; Zhou et al., 1995). In addition, the specific ablation of the gene for β-catenin in epidermis results in a deficiency in hair follicle formation (Huelsken et al., 2001), whereas non-degradable β-catenin expression in skin leads to de novo hair formation (Gat et al., 1998). Mice that lack Lef1, do not develop whiskers and show a reduced number of body hairs (van Genderen et al., 1994), and the increased expression of Lef1 in the epidermis of transgenic animals leads to defects in the positioning and orientation of hair follicles (Zhou et al., 1995). Finally, the expression of dickkopf 1, a potent diffusible inhibitor of Wnt action, in the skin of transgenic mice produces a complete failure of placode formation prior to morphological or molecular signs of hair differentiation (Andl et al., 2002). In addition to Wnt, a number of other key signaling pathways, including those modulated by fibroblast growth factors (FGFs) (du Cros, 1993; Ota et al., 2002; Rosenquist and Martin, 1996; Suzuki et al., 2000), bone morphogenetic proteins (BMPs) (Blessing et al., 1993; Botchkarev et al., 2001; Botchkarev et al., 1999; Botchkarev et al., 2002; Kulessa et al., 2000), TGFβ

(Foitzik et al., 2000; Foitzik et al., 1999; Paus et al., 1997) and Shh (Bitgood and McMahon, 1995; Chiang et al., 1999; St-Jacques et al., 1998), participate a reiterative manner during the hair follicle development (reviewed by Millar, 2002). More recently, different members of the TNFα receptor superfamily (Headon and Overbeek, 1999; Kojima et al., 2000; Koppinen et al., 2001; Laurikkala et al., 2001; Laurikkala et al., 2002; Mikkola et al., 1999; Monreal et al., 1999; Naito et al., 2002; Schneider et al., 2001; Thesleff and Mikkola, 2002) and subsequent signaling through NFκB family of transcription factors (Schmidt-Ullrich et al., 2001) have also been involved in hair follicle morphogenesis and cycling. Finally, the transcriptional co-activator p63 (reviewed by Brunner et al., 2002b; Irwin and Kaelin, 2001; Levrero et al., 2000; Marin and Kaelin, 2000; van Bokhoven and Brunner, 2002; Yang et al., 2002; Yang and McKeon, 2000) is of crucial importance for correct development of ectodermal appendages and mutations in the *P63* gene (*TP73L* – Human Gene Nomenclature Database) are found in a number of human syndromes that are characterized by defects in hair and teeth (reviewed by Brunner et al., 2002b; van Bokhoven and McKeon, 2002). This protein, the most recently discovered member of the p53 family, is expressed in embryonic ectoderm and in the basal, proliferative layer of epidermis (Parsa et al., 1999; Pellegrini et al., 2001), and appears to be a keratinocyte stem cell marker (Lee and Kimelman, 2002; Pellegrini et al., 2001). In agreement, besides other anomalies, the *p63*-knockout mouse lacks epidermis, apparently owing to the loss of stem cells required for the tissue (Mills et al., 1999; Yang et al., 1999). However, the actual molecular functions of p63 (Tcp1 – Mouse Genome Informatics) in hair growth and development have not been yet delineated.

Cell cycle withdrawal is a common prerequisite for terminal differentiation in most tissues. Consequently, those molecules implicated in cell cycle regulation might have additional functions modulating cell differentiation. This hypothesis has been clearly confirmed in the case of the retinoblastoma family of proteins. This includes pRb, p107 (Rbl1 – Mouse Genome Informatics) and p130 (Rbl2 – Mouse Genome Informatics). All these proteins modulate cell cycle progression during G1 through their ability to bind and inhibit different members of the E2f transcription factor family (Classon and Dyson, 2001; Weinberg, 1995), although with different affinity, as E2f1-3 bind preferentially to pRb, whereas E2f4 and E2f5 bind to p107 and p130 (Classon and Dyson, 2001). In addition, these proteins regulate different aspects of the differentiation process in a number of tissues (reviewed by Classon and Dyson, 2001; Lipinski and Jacks, 1999), as demonstrated by the analyses of mice lacking the different members of the retinoblastoma family. Rb-deficient animals die between day 13 and 15 of gestation, displaying overt defects in erithroid, neuronal and lens development (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). By contrast, mice deficient in p107 or p130 develop normally and do not display any overt phenotype (Cobrinik et al., 1996; Lee et al., 1996), indicating that, in most tissues, either p107 or p130 is dispensable for development, and suggesting a functional overlap between them. In agreement, mice that lack both proteins die immediately after birth and display defects in bone development, associated with impaired chondrocyte differentiation (Cobrinik et al., 1996). However, Rbl1/p107-deficient embryos die earlier than their *Rbl1*$^{-/-}$ littermates supporting the notion that p107 can substitute some of the functions of pRb in differentiation, allowing the extended survival observed in Rbl1-deficient embryos (Lee et al., 1996). Interestingly, the developmental consequences of p107 or p130 deficiency might be determined by the genetic background (LeCouter et al., 1998; LeCouter et al., 1996).

With respect to the possible functions of the retinoblastoma family in skin, we have reported that p107 and p130 are differentially expressed and participate during in vitro human keratinocyte terminal differentiation process (Paramio et al., 1998; Paramio et al., 2000). To investigate in more detail the relevance of the functionality of these proteins in epidermal differentiation in vivo, we have analyzed the skin in mice lacking p107 and/or p130. Our present results confirm that p107 and p130 are necessary to proper epidermal terminal differentiation and, in addition, are essential mediators in the inductive signals between epithelium and mesenchyme and important regulators of several morphogens involved in such inductive interactions.

MATERIALS AND METHODS

Mice and histological procedures

The mice with the different genotypes were generated and identified by PCR as described (Cobrinik et al., 1996; Lee et al., 1996). Skin samples or whole embryos at 14.5, 16.5 and 18.5 dpc (days post coitum) from wild-type and p107/p130-null mice were fixed in 10% buffered formalin or 70% ethanol, and embedded in paraffin wax. Sections (5 μm) were stained with Hematoxylin/Eosin or processed for immunohistochemistry and analyzed with a Zeiss Axiophot microscope.

Immunohistochemical methods

Immunohistochemistry was performed on deparaffinized sections using antibodies against K10 (K8.60 mAb, 1/75 dilution, Sigma), loricrin (1/500 dilution of a monospecific rabbit polyclonal antibody, Covance) and filaggrin (1/500 dilution of a monospecific rabbit polyclonal antibody, Covance). ΔNp63 was detected using 4A4 mAb (1/150 dilution; Santa Cruz Biotechnology) in formalin-fixed samples. Bmp4 and noggin antibodies were purchased from Santa Cruz Biotechnology (1/50 dilution), p75NTR (Ngfr – Mouse Genome Informatics) from Covance (1/500 dilution) and Hgf from R&D (1/50 dilution), and were used in formalin-fixed samples after microwave treatment. The expression of EDAR and XEDAR (R&D) and TROY/TAJ (Santa Cruz Biotechnology) (1/100 dilution) was monitored in ethanol fixed tissue sections. Proliferation was monitored by PCNA staining using PC10 mAb (generous gift of Dr D. P. Lane). p107 and p130 were detected in formalin-fixed sections using specific rabbit polyclonal antibodies (1/150 dilution; Santa Cruz Biotechnology). Horseradish-labeled secondary antibodies were purchased from Jackson Immunoresearch Laboratories and used 1:2000 dilution. The positive staining was visualized using Vector DAB kit, and slides were counterstained with Hematoxylin. Negative control slides were obtained by replacing the primary antibody with PBS (data not shown).

Northern blotting

Total RNA from frozen skin samples was isolated by guanidine isothyocianate-phenol-chloroform extraction. Northern blots containing total RNA (15 μg/lane) were probed for expression of the different signaling molecules using full-length cDNA as probes. The membranes were also hybridized with a 7S RNA probe to verify that equal amounts of mRNA were loaded and transferred.

Mouse skin grafts

Dorsal full thickness skin pieces of 2-3 cm^2 were obtained from 18.5 dpc p107/p130-null mice or double heterozygous littermates as control. Donor skin pieces were grafted onto a wound created by removing a similar-sized piece of full thickness back skin in female immunodeficient *NOD/scid* (*Prkdc* – Mouse Genome Informatics) recipient mice. Graft and host skin edges were joined using surgical silk suture and the grafted area was covered with a thin layer of NewSkin (Medtech, Jackson) as the only protective dressing. This procedure allows graft-take monitoring and produces normal-haired donor skin. Graft recipient animals were routinely monitored for hair growth and sacrificed 2-8 weeks after grafting. The graft-containing area was excised and processed for histopathology or immunohistochemical analysis as described above.

Band shift analysis

Electrophoretic mobility shift assays (EMSA) were performed by incubating whole-cell extracts from mouse skin with a labeled oligonucleotide corresponding to a palindromic κB: 5′-GATCCAA-CGGCAGGGGAATTCCCCTCTCCTTA-3′.

Complexes were separated on 5.5% native polyacrylamide gels in 0.25× Tris-borate-EDTA buffer, dried and exposed to Hyperfilm-MP (Amersham) at –70°C. The composition of the κB complexes in newborn mouse skin has been previously described (Budunova et al., 1999; Perez et al., 2000).

RESULTS

Altered epidermal terminal differentiation in p107/p130 double null animals

Mice deficient in p107 and p130 die after birth and display defective endochondral development (Cobrinik et al., 1996). However, no phenotypic alterations have been yet described in vivo in the skin of these animals. Given the involvement of the Rb and E2f families in differentiation of cultured human keratinocytes (Paramio et al., 1998; Paramio et al., 2000), we have analyzed the epidermis of p107 and/or p130 knockout mice. No alterations were found in the epidermis of animals lacking p107 or p130 compared with heterozygous littermates (data not shown), indicating that, as in other tissues, there exists compensation among the different members of the retinoblastoma family (Cobrinik et al., 1996). On the contrary, the epidermis of animals lacking both p107 and p130 displayed several abnormalities. We observed a reduction in the number and size of keratohyalin granules suggestive of altered terminal differentiation of the epidermal keratinocytes (Fig. 1A,A′,B,B′). To confirm this aspect we monitored the expression of several terminal differentiation markers. We found a decreased expression of filaggrin (Fig. 1D,D′) and loricrin (Fig. 1E,E′) in p107/p130-deficient epidermis. Conversely, we found that K10, an early marker of the process, is expressed in the first suprabasal layer in the epidermis of all the animals irrespective of their genotype (Fig. 1C,C′). These results indicate that, in agreement with our in vitro previous data (Paramio et al., 1998), the absence of p107 and p130 impairs the normal process of epidermal terminal differentiation, but not the commitment of the process.

Delayed hair follicle and tooth development in p107/p130-null animals

In p107/p130-null skin, there is also a generalized decrease in the number of the hair follicles (Fig. 2A,A′,D). During their morphogenesis, hair follicles can be classified in different developmental stages (reviewed by Muller-Rover et al., 2001; Paus et al., 1999). Based on this classification, we also characterized a clear developmental delay in hair follicle formation (Fig. 2E). This delay was evident as soon as day 14.5 dpc, at which the number of hair germs was severely reduced (Fig. 2B,B′), and persisted at days 16.5 and 18.5 dpc (data not shown and Fig. 2A,A′). In mice, the pelage consists of different types of hair with different morphology that are also formed at different times during embryogenesis. Primary or tyolotrich (guard) are induced by day 14.5 dpc, whereas the morphogenesis of secondary or nontylotrich (awl and zigzag) hair follicles starts by day 16.5 dpc. The fact that the differences between control and double-deficient mice occur at these different embryonic stages (Fig. 2D) strongly suggested that the morphogenesis and development of both tylotrich and nontylotrich hair follicles is affected in p107/p130-null skin. In addition, a similar delayed development was also observed in the whiskers, specialized hairs of the murine snout (Fig. 2C,C′).

The hair follicle morphogenesis depends on several processes similar to those acting during development of tooth germs (reviewed by Jernvall and Thesleff, 2000), and in many cases the mutations that give rise to hair defects, such as those in Lef1 (van Genderen et al., 1994) or *tabby-downless* also display alterations in tooth (Headon and Overbeek, 1999; Laurikkala et al., 2001; Laurikkala et al., 2002; Miard et al.,

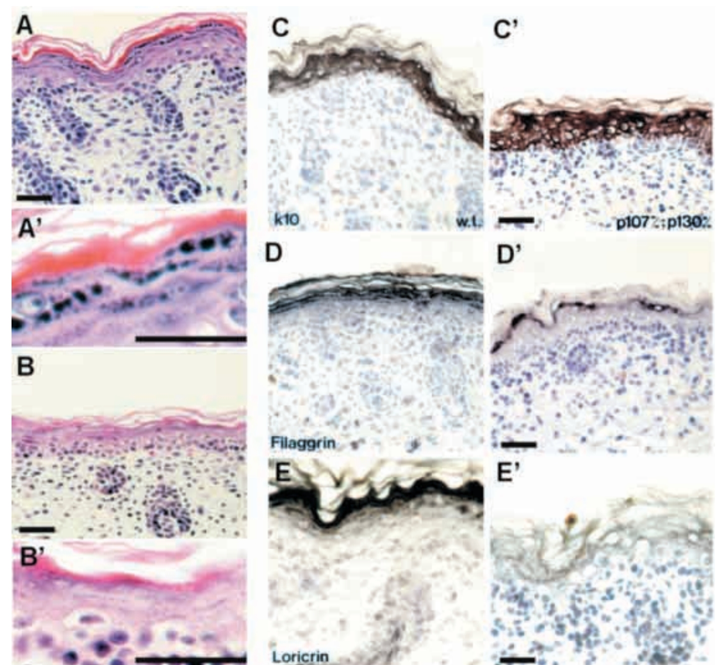

Fig. 1. Epidermal abnormalities and altered differentiation in p107/p130 knockout mice. Newborn skin sections of double heterozygous (A,A′) and p107/p130-null mice (B,B′). Note the decrease in the number and size of keratohyalin granules of the stratum granulosum in p107/p130-null animals (B′) compared with its respective littermate (A′). Expression of the epidermal differentiation markers in double heterozygous (C-E) and p107/p130-null animals (C′-E′) showing a similar expression of K10 (C,C′) and a decrease in the expression of the terminal differentiation-markers filaggrin (D,D′) and loricrin (E,E′). (A,A′,B,B′) Hematoxylin-Eosin staining. Scale bars: 100 μm.

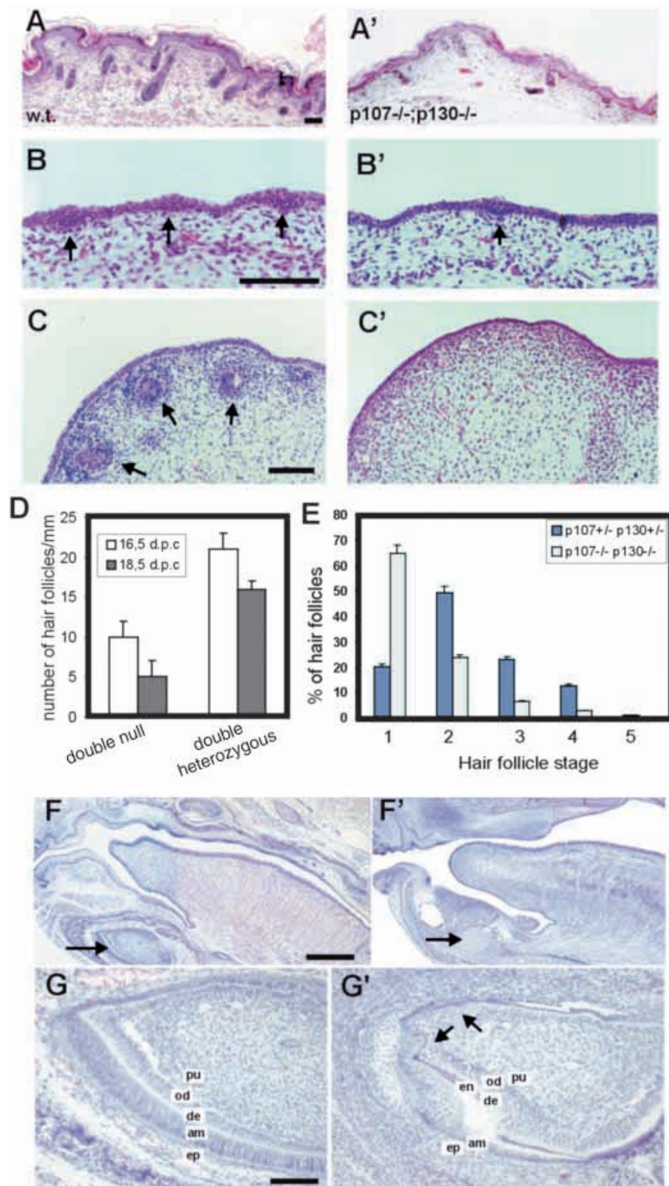

Fig. 2. Alterations in hair follicles and tooth germs in p107/p130 knockout mice. Hematoxylin/Eosin skin sections of wild type (A-C) and 107/130 null (A′-C′) 18.5 dpc (A,A′) and 14.5 dpc (B,B′) embryos showing the decrease in hair germs (see arrows). Snout of wild-type (C) and 107/130 null (C′) 14.5 dpc embryos showed a developmental delay of the whiskers. (D) Quantitative analysis of the number of hair follicles in the skin of 18.5 and 16.5 dpc embryos. (E) Analysis of the developmental stage of hair follicles in 18.5 dpc embryos. Data in D and E come from the quantification of three different sections of at least three different animals and are shown as mean±s.d. The hair follicle stages are as reported (Muller-Rover et al., 2001; Paus et al., 1999). Hematoxylin/Eosin medial sections from the heads of wild type (F,G) and 107/130 null (F′,G′) 18.5 dpc embryos. A few 107/130-null embryos showed anodontia with the presence of undifferentiated mesenchimal tissue in the site of incisors (compare arrows in F′ with those in F). When present, tooth germ in 107/130-null embryos displayed variable degrees of microdontia associated with hypoplasia and disorganization of the odontoblast layer (compare arrows in G′ with those in G). pu, dental pulp; od, odontoblast layer; de, dentine; am, ameloblast layer; ep, enamel pulp. Scale bars: 100 μm in A,C,C′,G,G′; 50 μm in B,B′; 500 μm in F,F′.

1999; Pispa et al., 1999; Thesleff and Mikkola, 2002). Consequently, we have analyzed the possible alterations in the development of tooth germs in p107/p130-deficient mice. We commonly observed severe alterations characterized by incisors microdontia (Fig. 2G,G′) and a few cases of anodontia, which in the most extreme situation leads to the complete absence of the incisors (arrows in Fig. 2F,F′). In addition, when present, tooth germs also displayed severe histological defects such as hypoplasia of the odontoblast layer (od in Fig. 2G,G′), with poorly differentiated and disorganized cells (arrows in Fig. 2G′, compare with 2G).

Many of the alterations found in p107/p130-deficient mice were similar to those found in mice bearing mutations in TNFα-like superfamily mediated signaling, including Eda-Edar, Xedar, Troy, etc. (Headon and Overbeek, 1999; Laurikkala et al., 2001; Laurikkala et al., 2002; Miard et al., 1999; Pispa et al., 1999; Thesleff and Mikkola, 2002) or in the downstream NFκB transcription factor family (Schmidt-Ullrich et al., 2001); consequently, we analyzed the expression of the receptors Edar (Fig. 3A,A′), Xedar (Fig. 3B,B′) and Troy/Taj (Fig. 3C,C′) in control and in p107/p130-deficient epidermis at 18.5 dpc. However, no differences were observed between control and mutant hair follicles. In agreement, EMSA analysis also showed similar endogenous NFκB activity among the different genotypes (Fig. 3D).

Collectively these observations indicate that the absence of the retinoblastoma relatives p107 and p130 altered the development of some specialized organs that depends on inductive interactions between epithelium and the underlying mesenchyme; this effect does not seem to be related to alterations in Eda/Edar or NFκB-dependent signaling.

Hair formation in p107/p130-deficient skin transplants

The early death of p107/p130-deficient mice precludes the analysis of epidermis and epidermal appendages in adults and the phenotypic evolution of the observed defects. To avoid this problem, we grafted p107/p130 epidermis onto *NOD/scid* mice. Hair growth in these transplants was evident four weeks after grafting in control and in p107/p130-deficient transplants (Fig. 4A,A′, respectively). Histological analysis demonstrated that, primary and secondary hairs are formed in both (Fig. 4B; data not shown). In p107/p130-deficient grafts, most of the hairs were morphologically normal with no alterations in any of the different cell populations (Fig. 4B). In addition, most anagen hair bulbs (Fig. 4C′) were indistinguishable from those of controls (Fig. 4C). However, a number of several abnormalities were also frequently detected in p107/p130-deficient transplants. First, we noticed that four weeks after grafting the number of hairs (Fig. 5A,A′) exceeded that of controls (Fig. 5B) by an average of three times. In addition, a severe interfollicular hyperplasia, which is in some areas associated to parakeratosis, was observed in mutant transplants (Fig. 5C). Secondly, besides the formation of normal hairs, we also noticed multiple hair abnormalities such as multiple hair follicles sharing a unique hair channel (Fig. 5D), twisted hair follicles lying in parallel to the epidermal surface (Fig. 5E), multiple follicular keratin-filled cysts (Fig. 5F), and hyperplasic sebaceous glands (Fig. 5G). Finally, we also observed a major number of follicles in anagen phase compared with controls (six times increase, on average). To

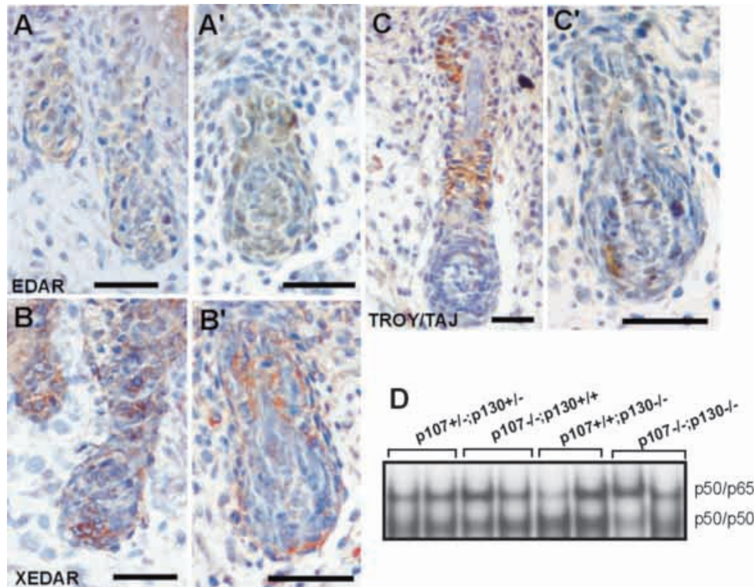

Fig. 3. Normal expression of Edar, Xedar and Troy/Taj and NFκB signaling in p107/p130 knockout mice skin. Immunohistochemical detection of Edar (A,A′), Xedar (B,B′) and Troy/Taj (C,C′) in hair follicles of 18.5 dpc control (A-C) and mutant (A′-C′) embryos showing no alterations. Scale bars: 50 μm. (D) EMSA analysis for NFκB binding activity of skin protein extracts from mouse of the quoted genotypes, showing no differences between them.

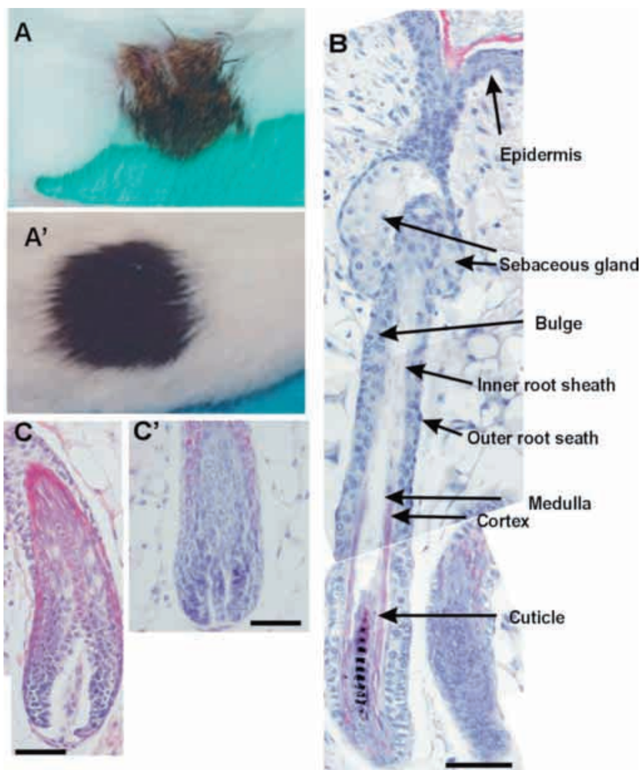

Fig. 4. Normal hair growth in grafts of p107/p130-null epidermis. Growth of hair coat in the grafts from control (A′) and p107/p130-doubly deficient mice (A) was evident four weeks after transplantation. Sections of skin grafts from p107/p130-doubly deficient mice (B,C′) showed normal anagen hair follicles without alterations in any of the different epithelial layers (B) and hair bulbs (C′) undistinguishable from those of control transplants (C). Scale bars: 50 μm.

monitor if hair cycling was affected in p107/p130-deficient transplants, we analyzed grafts at different times after transplantation. We observed that the vast majority of hairs formed 2 and 3 weeks after transplantation were already in anagen phase (Fig. 5H,I, respectively); however, owing to the severe inflammation, which is associated to the process of wound healing, we can not directly attribute this alteration to intrinsic abnormalities. Conversely, we observed that 8 weeks after grafting, while the vast majority of hairs in control samples were in resting telogen phase (Fig. 5J); in mutant samples almost all hairs were still in anagen phase (Fig. 5K). Taken together, these results indicate that, although the formation of normal hair occurs in p107/p130 grafts, a number of abnormalities affecting hair morphogenesis and development, as well as hair cycling, also arise in the absence of these proteins.

As commented above, the morphogenesis of hair follicles occurs during embryogenesis and relies on a series of signals sent between the mesenchymal cells and the overlying surface epithelial cells that cause fate changes in both cell populations (reviewed by Fuchs, 1998; Fuchs et al., 2001; Fuchs and Raghavan, 2002; Hardy, 1992; Millar, 2002). In control skin, the expression of p107 and p130, besides interfollicular keratinocytes, was detected in several cell types of the hair follicle, including the epithelial outer and inner root sheaths and the cortex, as well as the mesenchymal dermal papilla cells (Fig. 6A,B). Consequently, the hair developmental abnormalities of p107/p130-deficient mice can thus be attributed to alterations in the epithelial and/or in the mesenchymal compartments, as both are defective in these proteins. However, graft experiments can also help to discriminate between these possibilities. In fact, we observed that p107 and p130 proteins were expressed in the dermal papilla of hair follicles in null grafts (Fig. 6C,D), indicating that fibroblasts from the recipient reconstituted a follicular dermal papilla precursor, allowing the donor epithelium to develop into a mature anagen hair follicle. Therefore, the hair follicle abnormalities found in mutant skin are probably due to a defect in the mesenchyme that is restored in the transplants.

Altered keratinocyte differentiation and proliferation in the interfollicular epidermis of p107/p130-deficient skin transplants

The severe epidermal hyperplasia found in transplants (Fig. 5B; Fig. 6C,D,E) suggests altered differentiation of the interfollicular keratinocytes. To confirm this suggestion, we studied the expression of several epidermal differentiation markers. We observed that keratin K5 expression, which is confined to the basal layer in control grafts (not shown), was expanded in p107/p130-null grafts (Fig. 7A). K10 was uniformly present in all the suprabasal cells of control grafts, whereas in mutant transplants K10-negative areas were detected in the suprabasal layers (Fig. 7B). The keratin K6, which is expressed only in the hair follicles in normal adult epidermis as in control grafts (not shown), was present not only in the hair follicles but also throughout all the suprabasal layers in interfollicular epidermis of p107/p130-null grafts

(Fig. 7C). Finally, loricrin and filaggrin expression were severely decreased in p107/p130-deficient grafts (Fig. 7D; data not shown). Alterations in epidermal differentiation are commonly associated with altered proliferation. To study this, PCNA staining was performed in the transplants. We observed clear nuclear staining in control grafts four weeks after transplantation (Fig. 7E) that never exceeded 15% of the basal cells. However, in p107/p130-deficient transplants almost all the basal cells of the epidermis displayed a clear strong PCNA staining (Fig. 7F). The labeling also was clearly increased in normal and aberrant hair follicles (Fig. 7F). In addition, we also detected clear PCNA expression in suprabasal cells of the interfollicular epidermis (Fig. 7F′).

Collectively, these data demonstrate that the absence of p107 and p130 leads to impaired differentiation and increased proliferation in epidermal keratinocytes.

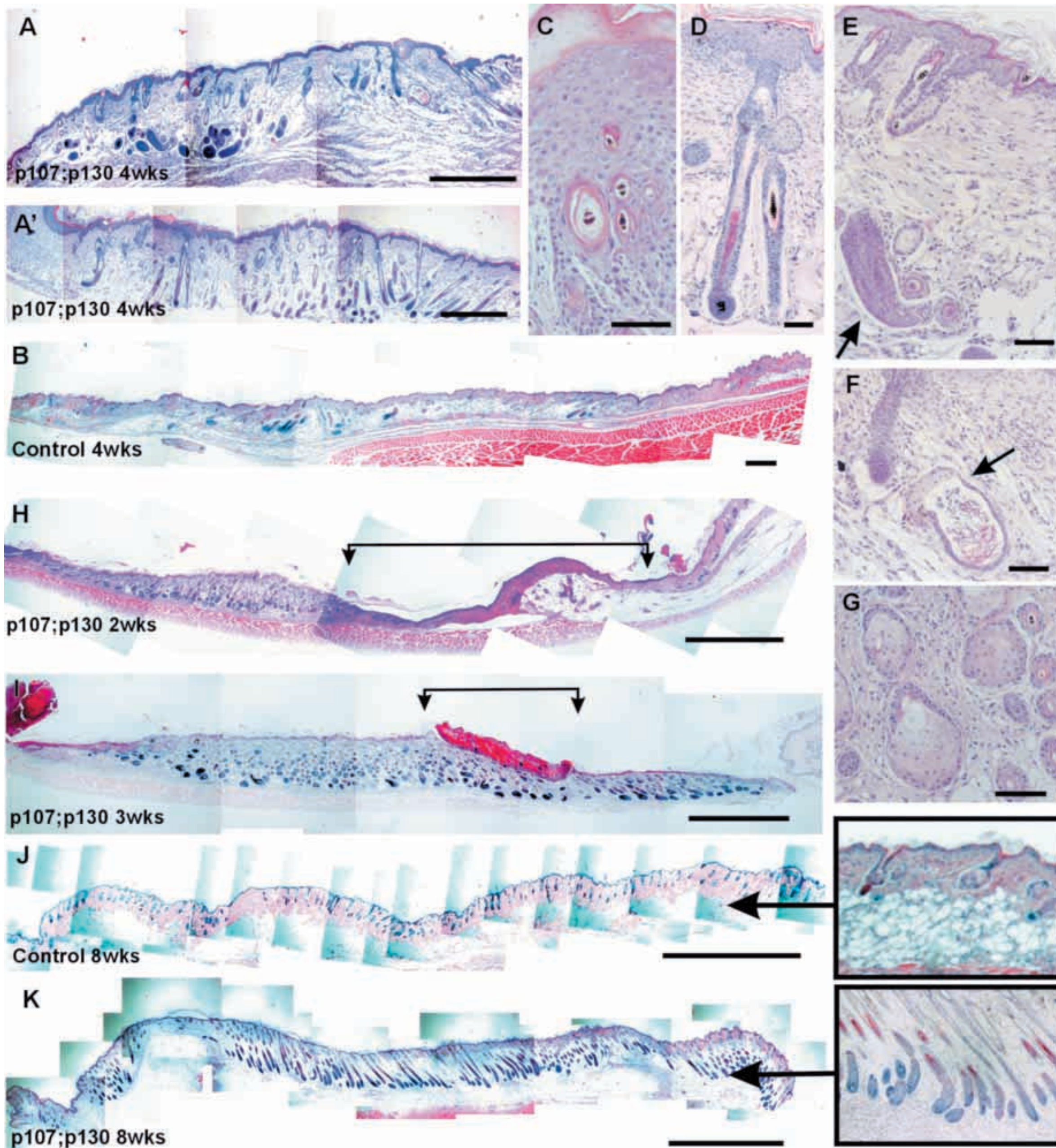

Fig. 5. Alterations in epidermis, aberrant hair follicle morphogenesis and hair cycling in grafts of p107/p130-null epidermis. Increased number of hair follicles in p107/p130-doubly deficient mice grafts (A,A′) compared with controls (B) was evident 4 weeks after transplantation. In addition, a number of abnormalities, including epidermal hyperplasia and parakeratosis (C), dysplastic hairs sharing a unique hair canal (D), misoriented follicles (arrow in E), follicular cysts (arrows in F), and hyperplasic sebaceous glands (G) were commonly detected in p107/p130-doubly deficient grafts. Transplants from p107/p130- deficient skin almost exclusively displayed anagen hair follicles at 2 (H) and 3 (I) weeks after transplantation. Eight weeks after transplantation, control grafts (J) display most of the hair follicles in a resting telogen phase (see inset, right), while in p107/p130-deficient grafts (K), the vast majority of hair follicles persisted in anagen (see inset, right). Scale bars: 500 μm in A,A′; 1 mm in B,H-K; 100 μm in C-G. Brackets in H and J indicate the wound margin.

Altered expression of hair morphogens in absence of p107 and p130

Among the different signals required for normal hair development are Shh (Bitgood and McMahon, 1995; Chiang et al., 1999; St-Jacques et al., 1998) and the components of the Wnt signaling pathway (reviewed by Fuchs et al., 2001; Fuchs and Raghavan, 2002; Millar, 2002). We thus monitored their expression by northern analyses. No major alterations were found in the expression of Shh, β-catenin, axin, Lef1 and Fwd1

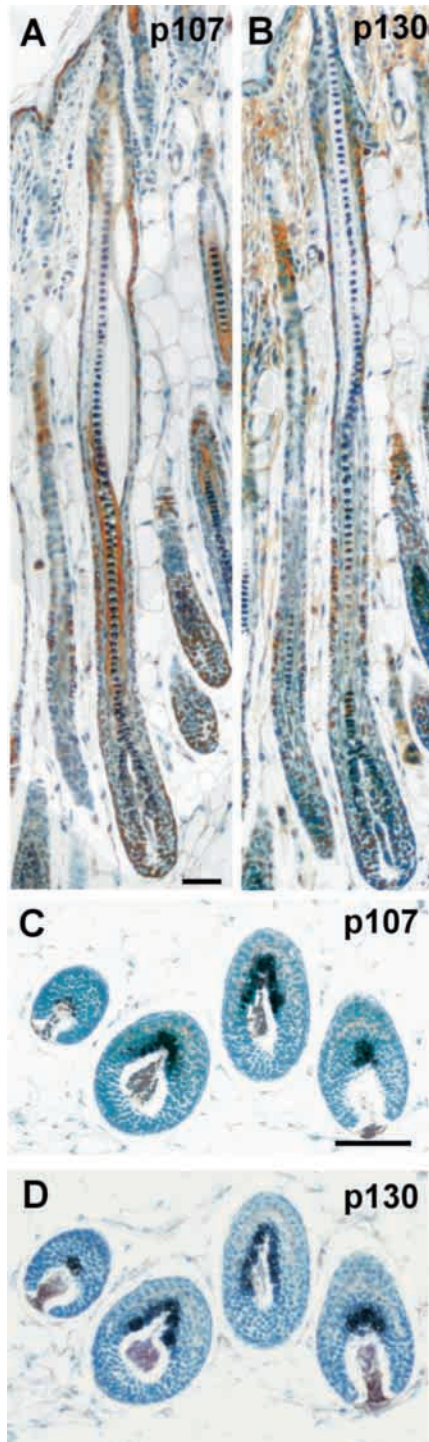

Fig. 6. Expression of p107 and p130 in anagen hair follicles. Immunohistochemical detection of p107 (A,C) and p130 (B,D) in control anagen hair follicles (A,B) and in hair bulbs from p107/p130-doubly deficient grafts (C,D). Note that in mutant grafts p107 (C) and p130 (D) immunoreactivity is detected in the nuclei of fibroblast forming the dermal papilla. Scale bars: 50 μm.

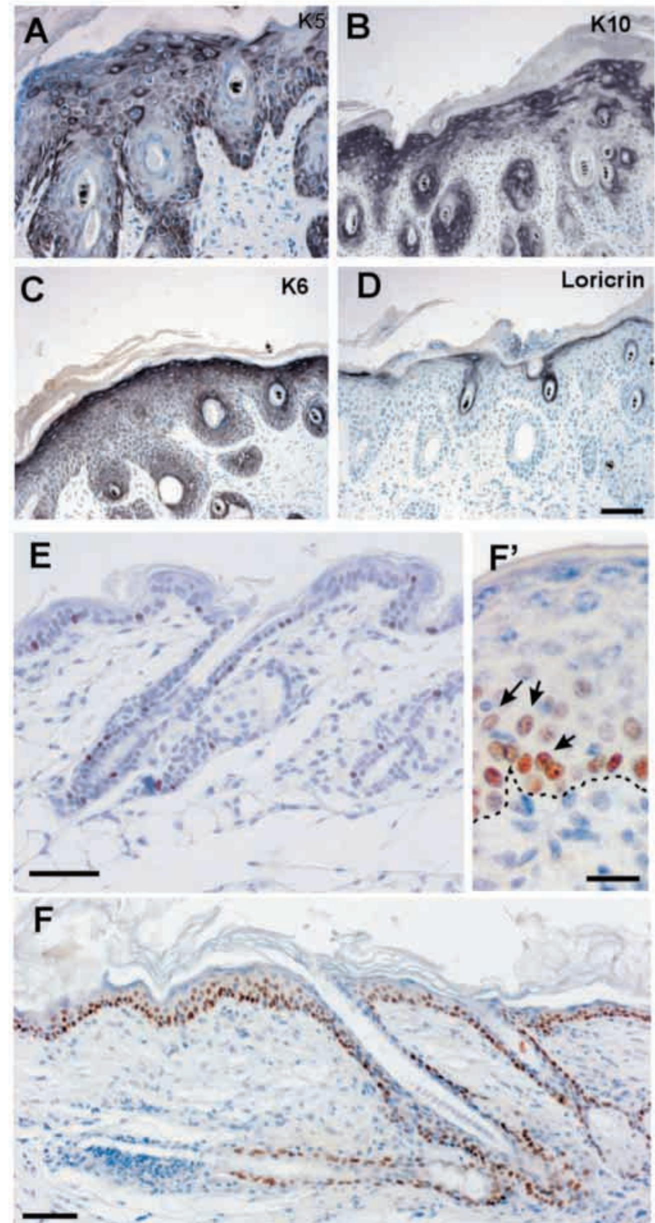

Fig. 7. Altered differentiation and proliferation of keratinocytes in transplants of p107/p130-deficient skin. K5 was expressed not only at the basal layer but also in scattered suprabasal cells (A). K10 was not expressed in the expected continuous suprabasal pattern (B). K6 was expressed in suprabasal layers of interfollicular epidermis (C). Loricrin expression was dramatically reduced (D). Proliferation was confined to a few keratinocytes in the basal layer of interfollicular epidermis and in the outer root sheath of hair follicles in control grafts (E), whereas the vast majority of these cells are actively proliferating in p107/p130-doubly deficient grafts (F). In addition, proliferating cells were also detected in some cells of the suprabasal layer of interfollicular epidermis in mutant transplants (arrows in F′). Scale bars: 100 μm in D,E; 200 μm in F; 25 μm in F′.

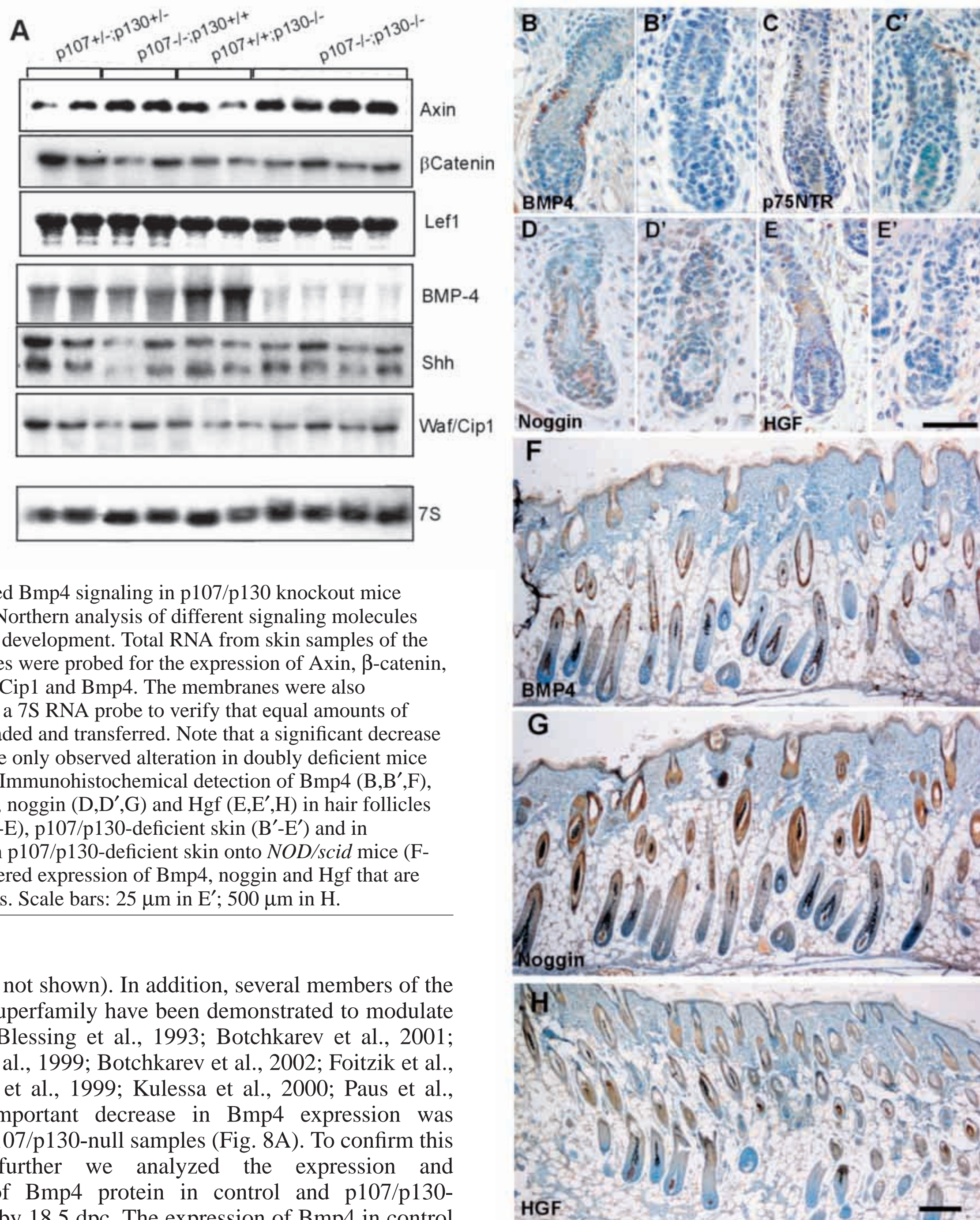

Fig. 8. Decreased Bmp4 signaling in p107/p130 knockout mice epidermis. (A) Northern analysis of different signaling molecules involved in hair development. Total RNA from skin samples of the quoted genotypes were probed for the expression of Axin, β-catenin, Lef1, Shh, Waf/Cip1 and Bmp4. The membranes were also hybridized with a 7S RNA probe to verify that equal amounts of mRNA were loaded and transferred. Note that a significant decrease in Bmp4 was the only observed alteration in doubly deficient mice samples. (B-H) Immunohistochemical detection of Bmp4 (B,B′,F), p75NTR (C,C′), noggin (D,D′,G) and Hgf (E,E′,H) in hair follicles from control (B-E), p107/p130-deficient skin (B′-E′) and in transplants from p107/p130-deficient skin onto *NOD/scid* mice (F-H). Note the altered expression of Bmp4, noggin and Hgf that are restored in grafts. Scale bars: 25 μm in E′; 500 μm in H.

(Fig. 8A; data not shown). In addition, several members of the BMP/TGFβ superfamily have been demonstrated to modulate hair growth (Blessing et al., 1993; Botchkarev et al., 2001; Botchkarev et al., 1999; Botchkarev et al., 2002; Foitzik et al., 2000; Foitzik et al., 1999; Kulessa et al., 2000; Paus et al., 1997). An important decrease in Bmp4 expression was observed in p107/p130-null samples (Fig. 8A). To confirm this observation further we analyzed the expression and localization of Bmp4 protein in control and p107/p130-deficient skin by 18.5 dpc. The expression of Bmp4 in control skin (Fig. 8B) is in accordance with previous reports (Botchkarev et al., 2002; Kulessa et al., 2000). However, we failed to detect Bmp4 protein (Fig. 8B′) in the hair follicles of the mutant skin, in agreement with the northern results. In hair follicles, the Bmp4-dependent signaling is modulated by a number of different morphogens, including noggin, p75NTR (Ngfr – Mouse Genome Informatics), Lef1 and Hgf, whose expression is also affected by Bmp4 (Botchkarev et al., 2001; Botchkarev et al., 1999; Botchkarev et al., 2002; Botchkareva et al., 1999; Kulessa et al., 2000; Lindner et al., 2000; Zhao et al., 2000). We have thus analyzed the expression and localization of these molecules in control and in p107/p130-deficient hair follicles. We did not detect any significant changes in p75NTR and Lef1 expression in mutant hair follicles (Fig. 8C,C′; data not shown). By contrast, the expression and localization of noggin and Hgf were severely perturbed in mutant hair follicles when compared with controls (Fig. 8D,D′,E,E′). These results suggest that, in the absence of p107 and p130, there is a defective Bmp4-dependent signaling that affects hair follicle morphogenesis and development.

Finally, as in grafted p107/p130 skin, we have observed the rescue of the hair growth defect, the above suggestion would implicate that in these grafts Bmp4 signaling should be restored. In fact analysis of the expression of Bmp4, noggin

and Hgf in the transplants (Fig. 8F-H) demonstrate the appropriate expression of these molecules.

Decreased expression of ΔNp63 in p107;130 deficient mouse epidermis

We next investigated the possible consequences of the observed alteration in Bmp4-dependent signaling. Among the possible targets of Bmp4 signaling, ΔNp63 has been recently identified in zebrafish as an ectoderm-specific direct transcriptional target (Bakkers et al., 2002). Moreover, two main aspects of p63 are of particular interest for the present study. First, there is a clear association of *P63* mutations with different human syndromes characterized by altered deficient hair and tooth growth (reviewed by Brunner et al., 2002a; Brunner et al., 2002b; van Bokhoven and McKeon, 2002). Second, in normal epidermis and in hair follicles, the ΔNp63 variant is preponderant and appears restricted to cells with high proliferative potential and is absent from the cells that are undergoing terminal differentiation (Parsa et al., 1999; Pellegrini et al., 2001). Consequently, given the observed alterations in the morphogenesis of hair and tooth (Fig. 2) and in keratinocyte proliferation (Fig. 7), together with the altered Bmp4 signaling (Fig. 8), it is possible to speculate that altered ΔNp63 expression might occur in p107/p130 mutant skin. In control embryos at day 18.5, ΔNp63 expression was observed in the nuclei of all basal cells in the interfollicular epidermis and also in the rudimentary follicles (Fig. 9A). Interestingly, immunoreactivity in the positive cells is not homogeneous, as some cells display increased ΔNp63 (arrows in Fig. 9A). In addition, ΔNp63 expression decreases as the cells enter the differentiation program and migrate into the suprabasal layers (Fig. 9A) as previously reported (Parsa et al., 1999; Pellegrini et al., 2001). However, a severe reduction in the expression of ΔNp63 was found in p107/p130-deficient keratinocytes (Fig. 9B). Conversely, one would expect that, if the reduced ΔNp63 expression was related to delayed development of the hairs in p107/p130-deficient skin, such expression would be restored in the transplants, given the restored hair growth observed in them. In fact, immunohistochemical analyses corroborate such hypothesis as ΔNp63 was found to be normally expressed in the grafted skin (Fig. 9C).

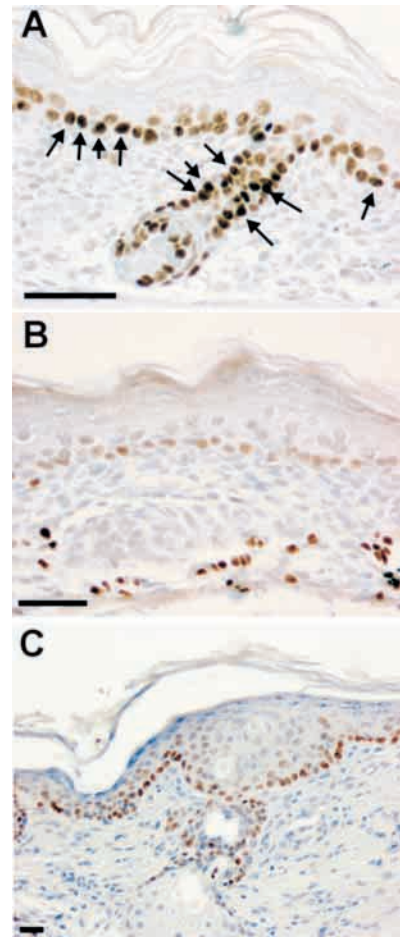

Fig. 9. Reduced expression of ΔNp63 in p107/p130 knockout mice epidermis is restored in transplants. The expression and localization of ΔNp63 were monitored in epidermis of control (A), doubly deficient mice (B) and skin grafts of doubly deficient mice on *NOD/scid* mice (C). Note that ΔNp63 expression is severely reduced in p107/p130-null epidermis and that this is restored in grafts. Scale bars: 25 μm.

DISCUSSION

The actual roles of retinoblastoma protein family in epidermis have not been yet fully determined. However, data obtained with cultured cells and in transgenic mice expressing modulators or targets of Rb family members, such as E2f (Pierce et al., 1998; Wang et al., 2001; Wang et al., 2000), viral oncogenes, such as HPV E7 (Greenhalgh et al., 1994) or E1A (Missero et al., 1993), or altered expression of cyclin-dependent kinase inhibitors (Di Cunto et al., 1998; Missero et al., 1995; Paramio et al., 2001) strongly support the existence of such roles. We have previously shown that the three pocket proteins are functionally involved in the process of in vitro differentiation of human keratinocytes, probably through the interaction with specific E2f members (Paramio et al., 1998; Paramio et al., 2000). We have focused on the roles of p107 and p130 in epidermis in vivo. We have found that the absence of any of these molecules do not cause phenotypic alterations, indicating that, as previously reported for other tissues (Cobrinik et al., 1996), there is functional compensation between these two proteins in skin. In agreement, the simultaneous absence of p107 and p130 produces severe skin abnormalities characterized by defects in epidermal terminal differentiation (Fig. 1) and reduced number and delayed morphogenesis of hair follicles (Fig. 2). Finally, we also show that the altered development of the hair follicle lies in defective interactions between the epithelium and the mesenchyme that also affect other organs requiring inductive interactions between these elements, such as whiskers and tooth germs (Fig. 2).

In agreement with our previous results (Paramio et al., 1998; Paramio et al., 2000), we found impaired terminal differentiation in absence of p107 and p130. At present, we do not know the molecular basis for such alteration; however, given that this seems to be a cell autonomous effect, as demonstrated in epidermal grafts, it is possible to speculate that this is mediated by direct interference with the epidermal

differentiation program. In fact, several epidermal-specific proteins are regulated by transcriptional elements such as Sp1 or C/EBP (Byrne et al., 1994; Kaufman et al., 2002; Kumar and Butler, 1999; Maytin and Habener, 1998; Maytin et al., 1999; Oh and Smart, 1998; Park and Morasso, 1999; Sinha and Fuchs, 2001; Zhu et al., 2002), which are themselves modulated by retinoblastoma family proteins (Charles et al., 2001; Chen et al., 1996; Classon et al., 2000; Decesse et al., 2001; Rohde et al., 1996; Udvadia et al., 1995). Conversely, we previously observed decreased E2f4 expression in terminally differentiating human keratinocytes, and most of the protein is bound by p107 and p130 (Paramio et al., 2000). Consequently, a possible explanation for the observed alterations would be that E2f4 expression facilitates epidermal differentiation commitment, whereas its expression at later stages would inhibit the process. However, recent data suggest that the mechanisms acting in mouse and human keratinocytes may differ, and complexes of E2f5 with p130 and histone deacetylases may be responsible for terminal differentiation of cultured mouse keratinocytes (D'Souza et al., 2001). The ongoing experiments will help to discern these possibilities and to determine the possible causes of such altered differentiation in epidermal keratinocytes as a consequence of p107 and p130 deficiency.

The altered development of the hair follicles and the incisors observed in p107/p130-deficient mice is of a particular interest. To our knowledge, this is the first evidence that these two proteins are involved in morphogenetic events. However, this observation is not totally surprising as E2f proteins mediate morphogenesis in Drosophila and Xenopus (Asano and Wharton, 1999; Hirose et al., 2001; Page et al., 2001; Suzuki and Hemmati-Brivanlou, 2000). More recently, the involvement of the retinoblastoma family in the control of morphogenesis has been highlighted by the finding that these proteins can form complexes with developmental factors that contain paired-like homeodomains (Wiggan et al., 1998). Interestingly, one of these factors, Alx4, displays an expression pattern that is restricted to sites of epithelial-mesenchymal interactions (Hudson et al., 1998; Qu et al., 1997a; Qu et al., 1997b). The possible functional relationship between p107 and/or p130 and Alx4 would merit future investigations.

The proper development of hair follicles and tooth require a large number of sequential interactions between the epithelial and mesenchymal cells that finally involve a tremendous plethora of different molecules belonging to several interconnected pathways (reviewed by Fuchs et al., 2001; Jernvall and Thesleff, 2000; Millar, 2002). The analysis of the expression of some of such molecules revealed a decreased expression of Bmp4 and altered Bmp4-dependent signaling (Fig. 8), but not in Eda/edar or in NFkB-depedent signaling (Fig. 3). We also found that, besides Bmp4, p107/p130-deficient hair follicles also displayed altered expression of noggin and Hgf, but not p75NTR (Fig. 8). Although at present we cannot rule out the possibility that these alterations, or those affecting other pathways regulating hair morphogenesis, might be the responsible for the findings in p107/p130-deficient mice, most of the phenotypic findings can be explained in terms of such diminished Bmp4 signaling.

Mice that lack the BMP antagonist noggin (Botchkarev et al., 2001; Botchkarev et al., 1999; Botchkarev et al., 2002) demonstrated that unregulated BMP proteins inhibit secondary hair development, show increased proliferation and reduced p75NTR, β-catenin and Lef1 expression in hair follicles, and also show reduced keratin K10 expression in the interfollicular keratinocytes. However, we observed that the absence of p107 and p130 inhibits both primary and secondary hair follicle development, without alterations in p75NTR, β-catenin and Lef1 expression (Fig. 8) and the effects on keratin K10 were only detected in deficient grafts (Fig. 7). Consequently, it is possible to discard noggin as a mediator of the phenotypic alterations found in skin of p107/p130-null mice. However, we found altered Hgf expression (Fig. 8). Whether this alteration might be upstream or downstream of Bmp4 signaling remains to be elucidated. In this regard, it is worth mentioning that both proteins are similarly expressed during *Xenopus* development (Aberger et al., 1997), but Bmp4, in contrast to Bmp2, cannot downregulate Hgf during limb bud morphogenesis (Scaal et al., 1999). The discrimination among these possibilities is a difficult task primarily due to the early embryonic lethality displayed by Hgf-deficient mice (Schmidt et al., 1995; Uehara et al., 1995). However, the fact that Hgf induces hair growth (Lindner et al., 2000; Shimaoka et al., 1995) would support a possible role for Hgf in the hair phenotype of the p107/p130-null mice. The functional relationship between BMPs and Hgf remains to be elucidated.

Bmp4 appears to play a dual role as an inhibitor and also an activator of hair and tooth development. Recent data also demonstrated that proper BMP signaling is required for appropriate hair formation and development (He et al., 2002; Kulessa et al., 2000). In agreement, Dlx3 and Msx2, which are activated by BMP signaling (Park and Morasso, 2002), are required for hair and tooth formation, and are associated with defective endochondral bone formation (Satokata et al., 2000). In this regard, it is worth mentioning that Bmp4 signaling is required for the maintenance of the differentiated postmitotic status of chondrocytes during endochondral ossification (Enomoto-Iwamoto et al., 1998; Minina et al., 2001), a process that is altered in p107/p130-null mice (Cobrinik et al., 1996).

The apparent opposite functions of Bmp4 during hair and tooth development might be due to different and sequential expression sites, either mesenchyme or epithelium, observed during the development of these organs, and which appear to be modulated by different transcriptional processes (Feng et al., 2002; Zhang et al., 2002). In particular, the expression in the mesenchyme seems to be modulated by sequences that contain putative sites for retinoblastoma and Sp1 binding (Feng et al., 2002; Zhang et al., 2002), which may account for the observed altered expression. In this regard, although we cannot discard that both the epithelium and the mesenchyme are responsible for the hair phenotype, the fact that in mutant skin grafts hair growth (Fig. 4) and the expression of Bmp4, noggin and Hgf (Fig. 8) are restored clearly points to mesenchymal defective signaling. This is further supported by the finding that dermal papilla cells in the mutant transplants express p107 and p130 (Fig. 6), suggesting that recipient fibroblasts have reconstituted a follicular dermal papilla precursor that allows the donor epithelium to develop anagen hair follicles.

Bmp4 signaling induces the expression of different target genes among which Lef1 and Waf/Cip1 (Jernvall et al., 1998; Kratochwil et al., 1996) appears to be highly relevant for hair and tooth formation. However, we do not detect decreased expression of any of these two genes in p107/p130-null mice

skin (Fig. 8 and data not shown). This is probably due to the fact that other factors can compensate the decreased Bmp4 expression or, alternatively, that such reduction is not enough to cause the repression of these genes. However, our data are in agreement with the increased Lef1 and decreased Bmp4 gene expression found using high-density oligonucleotide arrays to identify genes changed in response to activation of E2f species (Muller et al., 2001). More recently, ΔNp63 has been characterized as a transcriptional target of Bmp4 signaling (Bakkers et al., 2002). In agreement, we also detect decreased expression of ΔNp63 in the basal layer of the p107/p130-null epidermis (Fig. 9). Mice that lack the *p63* gene, are devoid of most epidermis and epidermal derivatives (Mills et al., 1999; Yang et al., 1999), and mutations in the *P63* gene are found in a number of human syndromes characterized by defects in hair and teeth (reviewed by Brunner et al., 2002a; Brunner et al., 2002b; van Bokhoven and McKeon, 2002). The possible causal relationship between such reduced ΔNp63 levels and the hair phenotype will be the subject of future investigation.

Collectively, we present here evidence for functional activities of the retinoblastoma family members in epidermis and epidermal appendages modulating not only differentiation and proliferation processes but also remarkably important morphogenetic events that lead to the formation of specialized ectodermal organs.

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