Roles for PDGF-A and sonic hedgehog in development of mesenchymal components of the hair follicle

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Accepted 31 March; published on WWW 19 May 1999

SUMMARY

Skin appendages, such as hair, develop as a result of complex reciprocal signaling between epithelial and mesenchymal cells. These interactions are not well understood at the molecular level. Platelet-derived growth factor-A (PDGF-A) is expressed in the developing epidermis and hair follicle epithelium, and its receptor PDGF-Rα is expressed in associated mesenchymal structures. Here we have characterized the skin and hair phenotypes of mice carrying a null mutation in the PDGF-A gene. Postnatal PDGF-A−/− mice developed thinner dermis, misshapen hair follicles, smaller dermal papillae, abnormal dermal sheaths and thinner hair, compared with wild-type siblings. BrdU labeling showed reduced cell proliferation in the dermis and in the dermal sheaths of PDGF-A−/− skin. PDGF-A−/− skin transplantation to nude mice led to abnormal hair formation, reproducing some of the features of the skin phenotype of PDGF-A−/− mice. Taken together, expression patterns and mutant phenotypes suggest that epidermal PDGF-A has a role in stimulating the proliferation of dermal mesenchymal cells that may contribute to the formation of dermal papillae, mesenchymal sheaths and dermal fibroblasts. Finally, we show that sonic hedgehog (shh)−/− mouse embryos have disrupted formation of dermal papillae. Such embryos fail to form pre-papilla aggregates of postmitotic PDGF-Rα-positive cells, suggesting that shh has a critical role in the assembly of the dermal papilla.

Key words: Hair, Development, Dermal papilla, Platelet-derived growth factor, Sonic hedgehog, Epithelial-mesenchymal interaction

INTRODUCTION

The development of many epithelial organs involves conserved patterns of inductive and permissive signaling between epithelial and mesenchymal tissues (Thesleff and Sahlberg, 1996), and in the mature organ epithelial and mesenchymal cells are generally tightly interconnected. This is true for the hair follicle, in which mesenchymal cells surround part of the follicle shaft (dermal sheath cells) and also build up the important dermal papillae, which provide the necessary signal(s) for proliferation and differentiation of follicular epithelial cells (Horne et al., 1986; Jahoda et al., 1984, 1993; Kollar, 1970; Oliver, 1967; Slee, 1962; Watson et al., 1994). In mice pelage hair follicles begin to develop on approximately embryonic day (E) 14. An essential event is the formation of aggregates of mesenchymal cells, pre-papillae, which remain at the base of the down-growing follicles. These aggregates subsequently become enclosed in epithelial pockets at the base of the follicles, at which site they are referred to as dermal papillae. Once the pre-papilla is formed below the follicular primordium, it does not seem to increase its cell number during follicular down-growth (Adelson et al., 1992; Orwin, 1979; Wessels and Roessner, 1965). The size of the dermal papilla appears to predict the thickness of the hair formed, at least the papilla size and follicle and hair fiber dimensions are correlated (Ibrahim and Wright, 1982; van Scott and Eckel, 1958). Hair thickness may thus be determined through regulation of the number of mesenchymal pre-papilla cells that aggregate. Moreover, an inverse correlation between hair thickness and hair follicle density suggests that the number of papilla cells is kept constant in relation to the skin surface area (Moore et al., 1998).

A number of growth and differentiation factors and their receptors are expressed in developing and/or mature hair follicles, and mutations in several of them affect hair formation. Thus genetic evidence suggests critical roles for FGF-, EGF-, hedgehog and wnt signaling in hair formation (Chiang et al., 1999; Gat et al., 1998; Guo et al., 1996; Hansen et al., 1997; Hébert et al., 1994; Lutetteke et al., 1994, 1993; Mann et al., 1993; St-Jacques et al., 1998). Platelet-derived growth factor-A (PDGF-A) and its receptor PDGF-Rα are expressed in cycling hair follicles and associated mesenchyme, respectively (Akiyama et al., 1996; Pontén et al., 1994), and a role for PDGF-A in hair follicle development has been suggested by injection of PDGF-Rα antibodies into newborn mice, which perturbed hair formation (Takakura et al., 1996). Homozygous null mutations in the known PDGF- (PDGF-A and PDGF-B) and PDGF receptor genes (PDGF-Rα and PDGF-Rβ) are all lethal. PDGF-B and -Rβ null mutants develop cardiovascular and renal abnormalities (Levén et al., 1994; Soriano, 1994), which, at least in part, reflect the failure of recruitment of mural cells to small blood vessels. In
RESULTS

PDGF-A+/− mice

PDGF-A+/− offspring that survive the E10 restriction point (Boström et al., 1996) are 10-30% smaller than heterozygous or wild-type siblings at birth, and gain weight at a slower rate postnatally. By 3 weeks and later, PDGF-A+/− mice are usually half the weight or less of control siblings. On 129/C57Bl6-hybrid genetic background we distinguished two categories of postnatal PDGF-A+/− mutants (Fig. 1). Category 1 mutants (more severe; Fig. 1A) were initially healthy, but soon failed to thrive and, unless killed, died spontaneously at around 2 weeks of postnatal age. Category 2 mutants (less severe) were clinically healthy over several weeks, appeared to have a normal repertoire of behavior, and passed through the weaning period without apparent problem (Fig. 1B). Unless killed, category 2 mutants died, usually at 4-6 weeks of age, from respiratory failure due to lung emphysema. The basis for the PDGF-A mutant phenotype variation is not understood but is

MATERIALS AND METHODS

Animals

PDGF-A+/− mice (Boström et al., 1996), PDGF-Rα+/− mice (Soriano, 1997) and shh+/− mice (Chiang et al., 1996) were all bred as 129/C57BL6 hybrids.

Histological analysis

Standard histological techniques were used. For immunohistochemistry (IHC), the following antibodies were used: anti-α-smooth muscle actin (ASMA) (clone 1A4; Dako); anti-desmin (clone D33; Dako); anti-vimentin (clone VIM-13.2, Sigma); biotin-conjugated anti-mouse Ig (Dako); anti-BrdU (#347580 Becton Dickinson); biotin-conjugated anti-mouse IgM (Dako); anti-vimentin (clone VIM-13.2, Sigma); anti-phospho-ERK (clone 1A4; Dako); anti-desmin (clone D33; Dako). The following probes were used: anti-Ptc (clone ASMA) (clone 1A4; Dako); anti-desmin (clone D33; Dako). The following probes were used: anti-Ptc (clone ASMA) (clone 1A4; Dako); anti-desmin (clone D33; Dako). The following probes were used: anti-Ptc (clone ASMA) (clone 1A4; Dako); anti-desmin (clone D33; Dako). The following probes were used: anti-Ptc (clone ASMA) (clone 1A4; Dako); anti-desmin (clone D33; Dako). The following probes were used: anti-Ptc (clone ASMA) (clone 1A4; Dako); anti-desmin (clone D33; Dako). The following probes were used: anti-Ptc (clone ASMA) (clone 1A4; Dako); anti-desmin (clone D33; Dako).

Skin transplantation

1 cm² pieces of full-thickness back skin were removed from E17.5 or E18.5 embryos and placed in ice-cold PBS. Equal size pieces of full-size skin were removed from the back of anesthetized male nude mice (nu/nu Balb/c) and the wounds were covered by the embryonic grafts and protected by sterile gauze and surgical tape.

Fig. 1. Skin and fur changes in PDGF-A+/− mice. Live-born mutants were of two categories. (A) Category 1 mutant at P7 with a control littermate. Inset shows mutant head. Note the presence of short pelage hairs but well-developed whiskers. (B) Category 2 mutant at P42 with control littermate. (C) Dissected P10 skin from category 1 mutant and control. Note the thinner and misshapen mutant hairs. (D) Dissected P25 skin from category 2 mutant and control. Note the abundance of second-hair-cycle anagen follicles in the control, whereas the mutant skin completely lacks anagen follicles, but has a sparse fur coat of first-cycle hairs in telogen phase. (E) Transplantation of wild-type and PDGF-A+/− skin to nude mice. (F) Wild-type transplant at 36 days post grafting. This graft contains first- as well as second-cycle hairs. (G) PDGF-A+/− transplant. Note the sparse and irregular growth of hairs. This graft contains numerous hairs that have failed to penetrate the epidermis.
Skin development in PDGF-A−/− mice likely to reflect genetic background differences. For the analyses described below, mutant animals were killed while they appeared clinically healthy.

Skin and hair defects in PDGF-A−/− mice

Category 1 mutants developed pelage hairs with about 2 days delay. Their hair coat was sparse and the hairs misshapen (Fig. 1A,C). Category 2 mutants developed first-hair-cycle hairs with normal timing, but second-cycle hairs, which normally appear around postnatal day (P)22, were never seen in mutants, the oldest animal being analyzed at P42. Dissections of the skin revealed high density anagen (growing phase) hair follicles in wild-type or heterozygous animals at P25 and P30, but a complete lack of anagen follicles in PDGF-A−/− skin older than P25 (Fig. 1D). Histological analysis confirmed these findings. At P25 and P30, PDGF-A−/− skin displayed only telogen (resting phase) follicles, whereas anagen follicles dominated in the skin of control siblings (Fig. 2C,D). First-hair-cycle anagen follicles in PDGF-A−/− mice were regionally crowded and misshapen and were smaller on average than the follicles in PDGF-A+/+ mice (Figs 2B, 3A, Table 1).

Fig. 2. Histology of PDGF-A−/− skin. Hematoxylin-erythrosin staining of skin sections from wild-type (A,C,E,G) and PDGF-A−/− (B,D,F,H) skin of indicated ages. (B) Category 1 mutant. (D,F) Category 2 mutants. (G,H) Skin grafts 36 days post grafting to nude mice. Broken lines mark the border between dermis (d) and subcutis (sc) (top line) and between subcutis and the skin muscle layer panniculus carnosus (pc) (bottom line). Note the reduction in thickness of the mutant dermis, which becomes dramatic at older ages (D,F). Mutant subcutis (region between the broken lines) is markedly thinner and contains hypotrophic adipocytes. Mutant skin contained many abnormally shaped hair follicles (e.g. arrow in B). The grafted PDGF-A−/− skin shows very poor organization, with fewer and severely misshapen hair follicles (e.g. arrows in H) and hyperplastic sebaceous glands (arrowheads in H). All micrographs are at the same magnification; bar, 1 mm.

The smaller size hair bulbs in PDGF-A−/− mice correlated with a decreased average thickness of hairs (Table 2).

Fig. 3. Abnormal follicular shapes in PDGF-A−/− skin. (A,B) P10 PDGF-A−/− mouse. Crowded and misshapen follicles (A) and inverted follicles (B). In B the base of the follicle is indicated by BrdU-labeled germinative epithelial cells. The follicle labeled with an arrow is inverted relative to the epidermis. (C,D) Grafted PDGF-A−/− skin. Note the abnormally shaped hair follicles (e.g. arrow in C) and hyperplastic sebaceous glands (asterisks). Bars, 100 μm.
normal deposits of brown fat at birth (data not shown). Category 1 mutants had substantially reduced subcutaneous deposits of white fat at P10 (Fig. 2A,B) whereas category 2 mutants initially had normal subcutaneous deposits that subsequently gradually disappeared (Fig. 2C-F). The loss of white adipose tissue in PDGF-A+/− mice was not restricted to the skin, but was seen also in the abdomen (data not shown). Vimentin antibodies labeled dermal adipocytes strongly in the PDGF-A+/− skin. In PDGF-A−/− skin, vimentin-positive cells reminiscent of adipocytes were seen but devoid of fat droplets (data not shown). Together, our observations imply that the loss of subcutaneous fat in PDGF-A−/− mice is not caused by a primary defect in adipocyte growth or differentiation. More likely, it reflects deficient fat storage, which may be secondary to respiratory failure or to malabsorption caused by intestinal villus dystrophy (Boström et al., 1996; L. Karlsson, P. Lindahl and C. Betsholtz, unpublished).

<table>
<thead>
<tr>
<th>Table 1. Number of cycling and non-cycling cells in the bulb of P10 wild-type and PDGF-A−/− follicles</th>
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<tr>
<td>Proliferating epithelial cells</td>
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<tr>
<td>Non-proliferating epithelial cells</td>
</tr>
<tr>
<td>Number of dermal papilla cells</td>
</tr>
<tr>
<td>Proliferating epithelial cells/total cells</td>
</tr>
<tr>
<td>Proliferating epithelial cells/dermal papilla cells</td>
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Numbers of BrdU-labeled and non-labeled cells were counted on sections (illustrated in Fig. 5) through 30 different bulbs for each genotype at the level of the dermal papilla. Numbers represent cells per section (not per bulb). Care was taken to count only follicles, which were cut through the center of the bulb. Only labeled and unlabeled cells in the area of the germinative epithelium were counted, and pigmented epithelial cells or cells external and adjacent to pigmented cells were excluded. Note that although the number of BrdU-labeled and non-labeled epithelial cells, as well as the number of dermal papilla cells, were smaller on average per PDGF-A−/− section, the relationships between the number of proliferating epithelial cells to total epithelial cells, and between papilla cell number and number of proliferating epithelial cells, were constant in wild-type and PDGF-A−/− follicles.

*Student’s unpaired one-tailed r-test.

Transplantation of PDGF-A−/− skin to nude mice
To control for systemic influences on hair development in PDGF-A−/− mice, skin transplants were performed to nude mice (Briggaman, 1985). PDGF-A−/− transplants showed a 2-3 day delay in hair outgrowth (not shown) and developed fewer and abnormally shaped hair follicles and hairs (Figs 1E-G, 2G,H, 3C,D). In contrast to the PDGF-A−/− mice, the grafted hair follicles entered the second hair cycle (data not shown). In the grafted PDGF-A−/− skin many hairs failed to penetrate the epidermis, and became cyst-like or zig-zag-shaped, and had associated hyperplastic sebaceous glands (Figs 2H, 3C,D, 4F). This was not seen in control transplants (Fig. 2G).

Loss of α-smooth muscle actin-positive dermal sheath cells in PDGF-A−/− mice and transplanted skin
We compared the expression of the smooth muscle cells (SMC) markers α-smooth muscle actin (ASMA) and desmin in PDGF-A+/+ and −/− skin since SMC have been shown to be dependent on PDGFs at other sites. In the skin, both ASMA and desmin were expressed in the arrector pili musculature and by vascular SMC (Fig. 4 and data not shown). ASMA was further expressed in a sharply demarcated region of the dermal sheath that surrounded the lower quarter of the hair follicle (Fig. 4A,C,E). Neither the dermal sheath cells at more superficial locations or surrounding the lower part of the follicle bulb, nor the dermal papilla cells, were ASMA-positive. Hair follicles in PDGF-A+/+ mice showed a significant reduction in the number of ASMA-positive dermal sheath cells (Fig. 4B,D). In transplanted PDGF-A−/− skin, the hair follicles were very heterogeneous with respect to the dermal sheaths, which were regionally thicker than normal and regionally missing (Fig. 4F and data not shown). Except for the dermal sheath, the ASMA and desmin expression patterns were similar in PDGF-A+/+ and −/− skin, indicating that other ASMA- or desmin-positive cells in the skin were not affected in PDGF-A mutants.

Reduced proliferation in PDGF-A−/− dermis
BrdU labeling showed that epithelial cells of the hair follicle bulb were actively proliferating in wild-type anagen hair follicles while the dermal papilla was postmitotic (Fig. 5A,B). At lower frequency, BrdU-labeled cells could also be identified in wild-type epidermis and in the dermal mesenchyme and in the region of the dermal sheath that was ASMA-positive (Fig. 5C and data not shown). Anagen PDGF-A−/− follicles showed less BrdU-labeled follicular bulb epithelial cells than wild-type follicles (Fig. 5D,E), but as these bulbs were also smaller, the labeling index was unchanged (Table 1). The number of proliferating bulb cells was equally proportional to the size of the dermal papilla in wild-type and PDGF-A−/− follicles (Table 1). In PDGF-A−/− skin the BrdU labeling was normal in the epidermis and in sebaceous glands but reduced in the dermal mesenchyme (Table 3) and in the dermal sheaths (Fig. 5F). During the first hair cycle anagen, terminal transferase-mediated dUTP-biotin nick end labeling (TUNEL) of wild-type and PDGF-A−/− skin sections revealed apoptosis in sebaceous glands only (data not shown). The decreased size of the mesenchymal components of PDGF-A−/− follicles was therefore not obviously connected to an increased apoptosis frequency at these sites during anagen.
Hair follicle initiation in PDGF-Rα—/— embryos
Theoretically, it is possible that maternal transfer of PDGF ligands may compensate for the loss of embryonic PDGF. It was therefore of interest to study hair follicle initiation in PDGF-Rα—/— embryos. Although many PDGF-Rα—/— embryos die at early gestation, a proportion of them live past E16 when bred as 129/C57Bl6 hybrids (Soriano, 1997). We studied two PDGF-Rα—/— E16.5 embryos that displayed the typical PDGF-Rα null phenotypes cleft face and spina bifida (Soriano, 1997). Histological examination of the skin showed that pelage hair follicle formation had initiated and proceeded normally for this age, including the formation of dermal

Table 3. Proliferation in the dermis and epidermis wild-type and PDGF-A—/— skin

<table>
<thead>
<tr>
<th>Genotype</th>
<th>BrdU-labeled epithelial cells*</th>
<th>BrdU-labeled dermal cells‡</th>
<th>Area counted (mm²)</th>
<th>Labeled dermal cells/mm²</th>
<th>BrdU-labeled dermal cells as % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1 (P10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>553</td>
<td>320</td>
<td>4.52</td>
<td>70.8</td>
<td></td>
</tr>
<tr>
<td>PDGF-A—/—</td>
<td>394</td>
<td>18</td>
<td>4.52</td>
<td>4.0</td>
<td>6</td>
</tr>
<tr>
<td>Experiment 2 (P10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>n.d.</td>
<td>175</td>
<td>2.86</td>
<td>61.2</td>
<td></td>
</tr>
<tr>
<td>PDGF-A+/—</td>
<td>n.d.</td>
<td>63</td>
<td>0.77</td>
<td>81.8</td>
<td>134</td>
</tr>
<tr>
<td>PDGF-A—/—</td>
<td>n.d.</td>
<td>92</td>
<td>3.91</td>
<td>23.5</td>
<td>38</td>
</tr>
<tr>
<td>Experiment 3 (P10)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>PDGF-A+/—</td>
<td>n.d.</td>
<td>899</td>
<td>5.25</td>
<td>171.2</td>
<td></td>
</tr>
<tr>
<td>PDGF-A—/—</td>
<td>n.d.</td>
<td>266</td>
<td>5.43</td>
<td>49.0</td>
<td>28</td>
</tr>
</tbody>
</table>

In experiments 1 and 2, the number of BrdU-labeled epithelial and mesenchymal cell nuclei were counted on sections from P10 PDGF-A—/— mice (category 1 mutants) and control littermates that had received a single BrdU injection and were killed 2 hours later. Areas covering the epidermis and dermis, but excluding the subcutis, were scored.

In experiment 3, a P10 PDGF-A—/— mouse (category 1 mutant) and a heterozygous control littermate received 5 consecutive BrdU injections at 2 hour intervals and were killed 2 hours after the final injection.

*Number includes epidermal cells and hair follicle cells. The latter include epithelial cells of the follicle shaft, sebaceous gland cells and possibly a small number of mesenchymal sheath cells.

†The majority of these cells were non-blood vessel-associated dermal mesenchymal cells. Less than 10% of the cells were associated with capillaries and may represent endothelial cells or pericytes.

n.d., not determined.
papillae (data not shown). In addition, whiskers were at an advanced stage of development (stage 6-8), and were histologically normal in PDGF-Rα-/− mice. Thus, hair follicle initiation took place normally and similarly in both PDGF-A-/− and PDGF-Rα-/− mice and was therefore judged to be independent of PDGF-Rα signaling.

Expression of PDGF-A and PDGF-Rα in developing skin and hair follicles
Hair follicle initiation takes place in three successive waves in the late prenatal period (E14-19) of murine development (Mann, 1962). We chose to study PDGF-A and PDGF-Rα expression between E15.5 and 17.5. Before the onset of hair follicle development, PDGF-A was strongly expressed in the basal layer of the epidermis, whereas PDGF-Rα was weakly expressed in the underlying mesenchymal dermis (Fig. 6A,B). Hardy (1992) has divided hair follicle development into stages. Stage 1-2 follicles showed strong PDGF-A expression (Fig. 6A). At this stage aggregates of PDGF-Rα-positive mesenchymal cells formed beneath the epidermal placode (Fig. 6B). These aggregates have the typical appearance of dermal pre-papilla. In stage 3-5 follicles, PDGF-A was strongly expressed in the hair follicle epithelium, particularly in the upper part of the follicle, but was downregulated, albeit still expressed at detectable levels, in the interfollicular epidermis (Fig. 6A). PDGF-Rα continued to be strongly expressed by the mesenchymal aggregates (pre-papillae), by mesenchymal cells surrounding the upper part of the hair follicle and by mesenchymal cells beneath the interfollicular epidermis (Fig. 6B). In PDGF-A-/− embryos, PDGF-Rα expression appeared normal before the onset of follicular morphogenesis and during stage 1-2 of follicular morphogenesis. In stage 3-5 follicles, however, PDGF-Rα-positive cells were reduced surrounding the upper part of the follicle, i.e. at the site where strong PDGF-A expression would normally occur (Fig. 6B).

Double labeling for PDGF-Rα mRNA and BrdU showed that the pre-papilla aggregates of PDGF-Rα-positive cells were postmitotic, similar to the dermal papillae of later stage follicles, whereas cycling PDGF-Rα-positive cells were otherwise frequent in the dermis (Fig. 7). This confirms the postmitotic state of dermal papillae from the beginning of their formation, and shows that their constituent cells are PDGF-Rα-positive from the earliest morphologically recognizable stage of pre-papilla formation.

Expression of shh, ptc and BMPs in developing skin and hair follicles
We next wanted to examine if the expression of other signaling molecules and receptors of putative importance in hair follicle...
Skin development in PDGF-A-/− mice

Fig. 6
patterning and early morphogenesis was affected in PDGF-A−/− skin. We focused on molecules that are presumptive activators or inhibitors of initiation of the morphogenesis of hair follicle and feather primordia. Shh has been reported to be expressed in developing hair follicle epithelium (Bitgood and McMahon, 1995; Iseki et al., 1996; Motoyama et al., 1998; Oro et al., 1997) and is a candidate activator of follicle morphogenesis. We found that both shh and ptc were expressed in the placode of stage 1 follicles (Fig. 6C). Ptc was also expressed in mesenchymal cells adjacent to the placode. During stage 2, shh expression was restricted to the lower part of the follicular epithelium and was typically polarized to the anterior portion of the bud (Fig. 6C and data not shown). Shh continued to be expressed in the epithelium of the lower part of the follicles at stage 3 and later, overlapping the region expressing ptc. Ptc was also expressed in mesenchyme surrounding the lower hair follicle epithelium during stages 2-3. Neither shh nor ptc expression patterns were affected in PDGF-A mutants (Fig. 6C).

Bone morphogenetic proteins (BMP)-2 and -4 are candidate inhibitors of feather and hair formation, both of which have been reported to be expressed by developing hair follicles (Bitgood and McMahon, 1995; Jones et al., 1991; Lyons et al., 1990). Here we found that BMP-2 was expressed in the placode during stage 1 and in the lower epithelium of stage 2 follicles. In stage 3 follicles, BMP-2 was expressed only in the epithelium adjacent to the dermal papilla (Fig. 6D). BMP-4 was expressed in the pre-papilla mesenchymal aggregates at stage 1, and later the expression appeared restricted to the papilla (Fig. 6E). No change in expression pattern of BMP-2 or -4 was noticed in PDGF-A mutant embryos (Fig. 6D,E).

**Abnormal hair follicle development and changed pattern of PDGF-Rα expression in sonic hedgehog−/− skin**

Shh and PDGF-A expression is overlapping in many developing organs including the hair follicle. Moreover, PDGF-Rα is generally expressed in mesenchymal cells adjacent to sites of expression of mammalian hedgehog proteins (L. Karlsson and C. Betsholtz, unpublished data). Since the PDGF-A−/− and shh−/− hair phenotypes are similar in the sense that the mesenchymal component of the follicles is affected (see below), we speculated that either of the PDGF-A/Rα and shh/ptc signaling systems may act in part by inducing the other. We found that shh/ptc expression appeared normal in PDGF-A−/− mice (see above), arguing that PDGF-A is unlikely to induce the shh/ptc system. The other possibility, that shh induces the PDGF-A/Rα system, was studied in shh−/− mice. Similar to recent reports (Chiang et al., 1999; St-Jacques et al., 1998), we found that shh−/− embryos displayed a block in hair follicle development at stage 2 (Fig. 6F and data not shown). Epidermal thickenings had formed that were reminiscent of placodes, but wider and less distinct morphologically than wild-type placodes. In shh−/− skin, the placodes expressed PDGF-A (Fig. 6G) and the dermal mesenchyme expressed PDGF-Rα (Fig. 6F), making it unlikely that shh acts by inducing the PDGF-A/Rα system. However, no dense aggregates of PDGF-Rα-positive mesenchymal cells were seen beneath the epithelial thickenings in shh−/− skin, implying that dermal pre-papillae had failed to form (Fig. 6F). In addition, general nuclear staining (DAPI) failed to reveal typical pre-papillae aggregates in shh−/− skin (data not shown). This suggests that shh has a role in the clustering of PDGF-Rα-positive mesenchymal cells beneath stage 1 follicles.

**DISCUSSION**

**A role for PDGF-A in the developing skin**

Based on a combination of expression patterns and genetic data we propose a model for PDGF-A function in skin development illustrated in Fig. 8. In the model, PDGF-A controls the proliferation of PDGF-Rα-positive dermal mesenchymal progenitor cells, which may differentiate into dermal papilla cells, into ASMA-positive dermal sheath cells, or into dermal fibroblasts. As discussed in more detail below, this suggested single function for PDGF-A in the skin is consistent with available genetic data (dermal phenotypes of PDGF-A−/− and PDGF-Rα-deficient mice (this study; Schatteman et al., 1992; Smith et al., 1991; Soriano, 1997; Stephenson et al., 1991) as well as the expression patterns of PDGF-A and -Rα in the developing skin and growing hair follicle (this study; Akiyama et al., 1996; Orr-Urtreger et al., 1992; Orr-Urtreger and Lonai, 1992; Pontén et al., 1994).

**PDGF-A and -Rα in the development of dermal mesenchyme**

Expression of PDGF-A in the epidermis and of PDGF-Rα in the underlying mesenchyme is seen before the onset of pelage formation.
hair follicle morphogenesis (Orr-Urtreger and Lonai, 1992; our unpublished results). At E14.5 and later, strong PDGF-Rα expression was seen in a few layers of mesenchymal cells subjacent to the epidermis. It is conceivable that these PDGF-Rα-positive cells are the targets for the epidermal PDGF-A. We show here that PDGF-A−/− mice develop dermal hypoplasia that becomes progressively more severe with age. BrdU labeling showed the dermal mesenchyme proliferated at a lower rate in PDGF-A−/− mice at early postnatal age. Together with previous in vitro studies that have established PDGF-A-containing PDGF dimers as potent mitogens for skin fibroblasts (Betsholtz et al., 1984; Östman et al., 1989), our results strongly suggest that PDGF-A acts by promoting the proliferation of dermal mesenchymal cells.

PDGF-Rα-deficient embryos show a variable but often severe loss of dermal mesenchyme leading to the formation of regional detachment of epidermis (blebs) in early embryos (Schatteman et al., 1992; Soriano, 1997). These severe defects are never seen in PDGF-A−/− embryos, implying that lack of embryonic PDGF-A may not lead to complete silencing of PDGF-Rα, and that our analysis of PDGF-A−/− mice underestimates the importance of PDGF-Rα signaling in the skin. Theoretically, loss of PDGF-A could be compensated for by PDGF-B, which has agonistic activity over PDGF-Rα, or by maternal transfer of PDGF-A to the embryo. PDGF-B is widely expressed by sprouting capillaries in the developing mouse, and is therefore locally present in the developing dermis (Lindahl et al., 1997). Whether maternal PDGFs could pass the placenta is unclear.

### Hair follicle formation

For the development of hair follicles, a reduction in the amount of dermal mesenchyme may influence epithelial-mesenchymal interaction. Our data show that the pre-papilla aggregate is composed of postmitotic PDGF-Rα-positive cells. We presume that these cells originate from the mitogenically active PDGF-Rα-positive cells underlying the epidermis, and that the cells become postmitotic in conjunction with cell clustering into pre-papillae. According to this reasoning, the PDGF-Rα expression by the dermal papilla cells may reflect the cells’ origin, rather than a function for PDGF-Rα in the dermal papilla. This conclusion is supported by our data showing that early hair follicle formation occurs normally in both PDGF-A−/− and PDGF-Rα−/− mice.

The smaller first cycle dermal papillae in PDGF-A−/− mice may reflect the fact that embryonic PDGF-A−/− skin contains a reduced number of PDGF-Rα-positive cells that aggregate into pre-papillae. Although a progressive reduction of the dermis is seen first postnataally, the reduced late embryonic and birth weights of PDGF-A−/− mice imply the presence of mesenchymal deficits already prenatally.

The failure of generation of second-cycle hairs in P25-42 PDGF-A−/− mice deserves further comment since this phenotype was not reproduced in the skin grafts. It is possible that systemic effects such as starvation or chronic disease in older postnatal PDGF-A−/− mice lead to a delay, or block, of the initiation of the second hair cycle. Hence, the second cycle block may not reflect a deficient local skin function of PDGF-A. However, the grafting experiments are not conclusive on this point, since systemic effects in the host may rescue the transplant. The surgical wound created likely leads to release of PDGF from host platelets and monocytes/macrophages, which may substitute locally for the PDGF-A deficiency of the graft. In addition, the intact PDGF-A−/− mouse and the graft are subject to different demands with regard to dermal proliferation. A 1 cm² piece of skin from a late embryo is normally expected to grow to cover at least a tenfold larger surface in a 25 day-old pup. In contrast, the same size graft shrinks due to wound contraction in the host to a size less than half of the original. If PDGF-A is an important mitogen for dermal mesenchymal progenitors, as we propose in our model, the intact PDGF-A−/− skin may become completely depleted of such progenitors in conjunction with postnatal skin growth, whereas the transplant may retain progenitors since there is less demand on dermal proliferation. Additional studies are therefore needed to address whether PDGF-A is needed for the initiation of the second hair cycle.

### Formation of the dermal sheath

The loss of ASMA-positive dermal sheath cells was noticeable...
in PDGF-A−/− mice, suggesting that PDGF-A is needed for the formation of a proper mesenchymal lining of the follicle shaft. PDGF-A−/− embryos show a reduction in the number of PDGF-Rα-positive cells lining the follicle shaft (Fig. 6B). These cells may be progenitors of dermal sheath cells. In contrast to the dermal papilla, the dermal sheath cells were not postmitotic, but cycled at much reduced frequency in PDGF-A−/− than in wild-type mice (Fig. 4). This suggests that PDGF-A produced by the follicular epithelium may drive the proliferation of dermal sheath cells during anagen. No specific function has previously been assigned to the dermal sheath. It is possible that the abnormal follicular shapes noticed in the PDGF-A−/− skin are connected to aberrant formation of dermal sheaths.

The fact that the dermal sheath cells affected in PDGF-A−/− mice are ASMA positive is interesting in relation to other phenotypes of PDGF-A and PDGF-B null mice. With the exception of oligodendrocytes (Calver et al., 1998; Fruttiger et al., 1999) these involve various subtypes of smooth muscle cells (SMC) or myofibroblasts. So far it has been found that alveolar SMC (Boström et al., 1996; Lindahl et al., 1997) and intestinal villus SMC (L. Karlsson, P. Lindahl and C. Betsholtz, unpublished) are deficient in PDGF-A−/− mice, whereas vascular SMC, in particular pericytes and mesangial cells, are deficient in PDGF-B−/− and PDGF-Bβ−/− mice (Levén et al., 1994; Lindahl et al., 1998, 1997; Soriano, 1994).

**Shh directs formation of the dermal papilla**

During the preparation of this paper, the hair phenotype of shh−/− mice was reported (Chiang et al., 1999; St-Jacques et al., 1998). These results showed that shh−/− hair follicles were blocked at stage 2. Grafting of shh−/− skin to nude mice resulted in the formation of abnormally shaped hair follicles with rudimentary dermal papillae and no formation of hair (Chiang et al., 1999; St-Jacques et al., 1998). Since mesenchymal follicle components are affected in both shh−/− and PDGF-A−/− skin, we investigated potential epistatic relationships between shh and PDGF-A signaling. Expression studies in PDGF-A−/− and shh−/− tissues seemed to rule out the simplest scenarios, i.e. that PDGF-A controls skin expression of shh or ptc, or vice versa, that shh controls the expression of PDGF-A or PDGF-Rα. However, the PDGF-Rα expression pattern was different in shh−/− skin, in that no clustering of PDGF-Rα-positive mesenchymal cells was evident beneath shh−/− follicles. Thus, it is possible that shh directly or indirectly controls clustering, or sorting, of papilla cells into distinctive and properly sized units needed for the correct anatomical organization of the anagen hair follicle. This could for instance be achieved by controlling the expression of cell adhesion molecules. Since the PDGF-Rα-positive cluster is postmitotic from its earliest recognizable stage, shh is probably not a mitogen for the dermal papilla cells. Moreover, since shh−/− skin shows the formation of rudiments with papilla characteristics, and some degree of epithelial differentiation in the follicular epithelium, shh is probably not involved in induction of the papilla cell fate (Chiang et al., 1999; St-Jacques et al., 1998).

We thank Sara Beckman for excellent technical assistance, Drs William Richardson, Brigid Hogan, Andrew McMahon, and Helena Edlund for DNA probes, Dr Philippe Soriano for PDGF-Rα−/− mice and Dr Chin Chiang for Shh+/− mice. The work was supported by grants from the Swedish Cancer Foundation, Medical Research Council (MFR), Göran Gustafssons Foundation, and IngaBritt and Arne Lundberg Foundation.

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