Control of thrombin signaling through PI3K is a mechanism underlying plasticity between hair follicle dermal sheath and papilla cells

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Summary
In hair follicles, dermal papilla (DP) and dermal sheath (DS) cells exhibit striking levels of plasticity, as each can regenerate both cell types. Here, we show that thrombin induces a phosphoinositide 3-kinase (PI3K)-Akt pathway-dependent acquisition of DS-like properties by DP cells in vitro, involving increased proliferation rate, acquisition of "myofibroblastic" contractile properties and a decreased capacity to sustain growth and survival of keratinocytes. The thrombin inhibitor protease nexin 1 (PN-1, also known as SERPINE2) regulates all those effects in vitro. Accordingly, the PI3K-Akt pathway is constitutively activated and expression of myofibroblastic marker smooth-muscle actin is enhanced in vivo in hair follicle dermal cells from PN-1−/− mice. Furthermore, physiological PN-1 disappearance and upregulation of the thrombin receptor PAR-1 (also known as F2R) during follicular regression in wild-type mice also correlate with such changes in DP cell characteristics. Our results indicate that control of thrombin signaling interferes with hair follicle dermal cells plasticity to regulate their function.

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Key words: PN-1, Thrombin, PI3K pathway, Cell plasticity, Cell differentiation, Hair follicle

Introduction
Hair follicles consist of an external epithelial envelope (outer root sheath; ORS) that is continuous with the epidermis, ensheaths the shaft and its thin internal envelope (inner root sheath, IRS) and embedded in it is a distinct group of specialized dermal cells, the papilla (DP). This core is itself surrounded by mesenchymal tissue, the dermal sheath (DS) (Paus and Cotsarelis, 1999). The hair follicle demonstrates a remarkable ability for self-regeneration, undergoing a regular succession of growth (anagen) and regression (catagen) phases associated with replacement of the old hair shaft by a new one (Hardy, 1992). This cyclic event requires particular interactions between the epithelial and the mesenchymal components of the follicle (Jahoda and Reynolds, 1996). The downgrowth of the follicle as well as the survival of cells in the epithelial sheaths are under the active control of signaling molecules produced by the papilla (Cohen, 1961; Jahoda et al., 1984; Reynolds et al., 1991). Cyclic shut down of this activity leads to disorganization, massive apoptosis and partial follicular regression initiated in the lower part of the follicle (Botchkarev and Kishimato, 2003). Thus, entry into new anagen requires the reactivation of DP activity, leading to stimulation of epithelial stem cells present in the permanent portion of the ORS to build a new functional follicle (Cotsarelis et al., 1990).

DP and DS cells are thought to derive from the same embryonic founder cells (Oliver, 1966). They are generated from mesenchymal cells in the embryonic dermis in response to signals generated by the epidermis (Hardy, 1992; Oro and Scott, 1998). DP cells are secretory cells that work together as an organizer, regulating growth and renewal of neighboring tissues. External DS cells provide structural reinforcement to the follicle. They express smooth muscle actin (Jahoda et al., 1991) and, thus, are able to generate strong contractile forces. Furthermore, DS cells located closest to the papilla, i.e. in the dermal sheath cup (DSC), exhibit an intermediate phenotype with both DP and DS characteristics (McElwee et al., 2003). They express smooth muscle actin and also a low level of alkaline phosphatase, considered to be a DP marker. Moreover, they can substitute for the DP in inducing the formation of new hair follicles when transplanted (McElwee et al., 2003).

The two dermal compartments are physically connected (Oliver, 1991; Jahoda, 1998) and a two-way traffic of cells between the DS and DP has been postulated to explain cyclic variation in DP size in the absence of detectable cell death and division (Elliott et al., 1999; Tobin et al., 2003). Indeed, in adult mice, DP cells are quiescent, whereas DS cells undergo sporadic proliferation (Pierard and de la Brassinne, 1975; Jahoda, 1998). Experimental evidence suggests that the DS is a cell reservoir for slow renewal of the papilla (Tobin et al., 2003). In addition, the capacity of grafted DSC cells to induce follicle formation probably relies on their ability to give rise to DP cells (McElwee et al., 2003). Conversely, DP cells participate in DS reconstitution in transplantation experiments, where some grafted cells are incorporated into newly formed DS (Jahoda, 1992; McElwee et al., 2003).

Overall, this points to a significant level of cell plasticity and suggests that change in the differentiation status of DP and DS cells might play a physiological role during the hair cycle. However, it is unclear whether variation in tissue size, cell renewal and cross-reconstitution of DP and DS rely on the presence of common
undifferentiated precursors (Lako et al., 2002; Jahoda et al., 2003; Fernandes et al., 2004) and/or on the transdifferentiation of mature mesenchymal cells. Despite the fundamental and the practical interest in understanding how two very specialized cell types can regenerate cells of the alternate phenotype, nothing is known about the molecular mechanisms or factors involved.

In situ hybridization experiments suggest that extracellular proteolytic activity is involved in the hair cycle. First, the protease-activated thrombin receptor 1 [coagulation factor II (thrombin) receptor, F2R; hereafter referred to as PAR-1] is expressed during the entire cycle in the DS, including a bridge of DSC cells invaginating into the DP area at the bottom of the follicle, but is present only during catagen in DP cells (Anan et al., 2003). Second, protease nexin-1 [also known as serpin peptidase inhibitor (SERPIN2), hereafter referred to as PN-1], a serine protease inhibitor targeting thrombin, plasminogen activators tPA and uPA, trypsin or plasmin (Baker et al., 1980; Guenther et al., 1985; Stone et al., 1987), is prominently expressed in hair follicle dermal cells during anagen, but is downregulated during catagen (Yu et al., 1995). Thus, the absence of PAR-1 and the presence of PN-1 in the DP during anagen appear to be a ‘double-lock’ which prevents thrombin-signaling activity on these cells. By contrast, the absence of inhibition by PN-1 and the upregulation of PAR-1 during catagen may allow efficient thrombin signaling through activation of its receptor. This suggests that the tight control of thrombin activity plays a role in the proper functioning of the DP. Thrombin can trigger fibroblast proliferation (Chambard et al., 1987) but can also promote their differentiation into myofibroblasts (Bogatkevich et al., 2001). These observations prompted us to investigate the role of PN-1-mediated thrombin inhibition on the renewal and differentiation of hair follicle dermal cells during the hair cycle.

In this study, we have established that control of thrombin signaling is a key component of hair follicle dermal cell plasticity. Using cultures of cells derived from PN-1–/– mice, we show that the inhibition of thrombin signaling antagonizes the acquisition of a DS-like phenotype by DP cells by preventing activation of the phosphoinositide 3-kinase (PI3K)-Akt pathway. Furthermore, we observe that PI3K pathway activation and expression of the myofibroblastic marker α smooth-muscle actin (SMA) are also altered in vivo by a change in the thrombin-PN-1 balance in both PN-1–/– mice and physiologically during anagen/catagen transition in wild-type animals.

Results
PN-1 colocalizes with thrombin and controls its activity in vibrissal follicles

To precisely monitor PN-1 expression during embryonic development and the cycle of hair/whisker follicles, a PN-1 reporter mouse showing bicistronic expression of β-galactosidase from the PN-1 locus (Kvajo et al., 2004) was examined using X-Gal staining and immunohistochemistry with an anti PN-1 antibody. PN-1 was first expressed at E16.5 in aggregated cells below the hair germ in a position corresponding to the presumptive DP (Fig. 1A). Expression then extended to the maturing DS as hair morphogenesis proceeded (Fig. 1B ‘anagen’ and 1C). In agreement with in situ hybridization data published by Yu et al. (Yu et al., 1995), secreted PN-1 was detected in and around its area of synthesis until catagen of the follicular cycle, was absent during catagen (Fig. 1B) but its synthesis resumed in DP and DS during renewed anagen (Fig. 1C).

In cycling follicles, PN-1 was also transiently expressed at the end of catagen in the ORS until the early phases of the new anagen (Fig. 1C). The tip of the growing hair progressed through these PN-1-expressing cells, which then surrounded both the growth cone of the new shaft and the club of the previous one (Fig. 1C).

Thrombin, the main PN-1 target, was also detected in whisker follicles by immunohistochemistry (Fig. 1D). It tightly colocalized with PN-1 both in DS and in a subset of ORS cells with the same spatiotemporal pattern (Fig. 1, compare D and C). Such colocalization allowed control of thrombin, illustrated by a twofold increase in thrombin-specific proteolytic activity measured in homogenates from PN-1–/– microdissected whisker follicles (Fig. 1E). This increased activity reflected the global absence of inhibition in all follicular tissues but appeared to be due more specifically to de novo accumulation of thrombin in the DP of PN-1–/– mice (Fig. 1F). This overload probably reflects a failure in the clearance of the protease by the low density lipoprotein receptor-related protein-1 (LRP-1), which controls internalization of PN-1-thrombin complexes (Knauer et al., 1997).

PN-1 limits thrombin-induced proliferation and acquisition of a myofibroblastic phenotype in cultured DP cells

Immunocytochemical analysis indicated that DP cells from wild-type anagen follicles homogeneously expressed PN-1 and LRP-1 in culture (Fig. 2A,B). NCAM and the DP-specific marker alkaline phosphatase [AP (Handjiski et al., 1994)] confirmed the identity and homogeneity of our cultures (Fig. 2D,E). BrdU incorporation indicated that the in vivo quiescent DP cells recovered the ability to divide when they were taken out of their niche and cultured as adherent cells in a proliferation-permissive medium containing FCS and EGF (Fig. 2F). Furthermore, as already observed (Jahoda et al., 1991; Reynolds et al., 1993), cultured DP cells also homogeneously expressed alpha smooth muscle actin (SMA; Fig. 2F), which is only produced in vivo in the dermal sheath compartment (Jahoda et al., 1991). Cultured DP cells thus had the combined characteristics of the DP and DS cell types. They nevertheless exhibited both lower proliferation rate and lower expression of SMA contractile protein than cultured DS cells (supplementary material Fig. S1A,B).

Except for PN-1 expression, the same markers were detected in DP cells from PN-1–/– follicles. However, these PN-1–/– DP cells exhibited a significantly higher proliferation capacity than those derived from wild-type littermates. The difference was quantified by counting cells at different time points after cell plating. The population doubling time of about 23 hours for wild-type cells was shortened to 15 hours for PN-1-deficient DP cells (Fig. 3A). In the FCS-supplemented medium used to culture those cells, about 0.05 U/ml of thrombin proteolytic activity was detected (data not shown). Inhibition of this activity by 100 μM hirudin for 12 hours triggered a significant decrease in DP cell proliferation, measured by BrdU incorporation, for cells of both genotypes. This effect was more pronounced for PN-1–/– cells, and hirudin treatment abolished the difference between the wild-type and mutant cells (Fig. 3B). Therefore, the proliferation of cultured DP cells was largely due to the presence of active thrombin in the culture medium and the lack of thrombin inhibition was responsible for the observed faster proliferation of PN-1-deficient cells.

Cells double in size before entering mitosis and mass increase is generally coordinated with, and somehow controls, cell division. However, the variation in size of quiescent DP cells in vivo suggests that these cells might uncouple size increase and cell division (Tobin et al., 2003). Consequently, we examined whether variations in proliferation rate were linked and proportional to changes in cell
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Cells were arrested in S phase using the DNA polymerase inhibitor aphidicolin and the increase in their volume, known to be proportional to the amount of newly synthesized material (Conlon et al., 2001), was measured. When tested in medium containing 1% serum over a 24-hour period, the absence of PN-1 caused a 2.5-fold increase in cell volume (Fig. 3C). It thus did not dissociate the two processes. Overgrowth of PN-1−/− cells was demonstrated to be directly due to lack of the protein, because it was abolished by addition of recombinant PN-1 (Fig. 3C). However, PN-1 internalization or signaling through LRP (Knauer et al., 1997) was not required. Blockade of PN-1 binding to the LRP receptor by the antagonistic receptor-associated protein RAP, used at a concentration interfering with proliferation (Vaillant et al., 2007), did not affect cell volume increase (Fig. 3C).

SMA protein level was higher in the absence of PN-1 both in lysates of cultured DP cells and in extracts from hairy skin (Fig. 4A). This correlated with a change in contractile properties of cultured cells. Wild-type DP cells embedded in a 3D collagen gel matrix, promoted a mild 10-15% contraction of the gel over 48 hours. By contrast, PN-1−/− cells caused an efficient 30-40% contraction of the collagen matrix (Fig. 4B). Thrombin activity was found to be responsible for stimulated contractile properties of DP cells. Indeed, a daily hirudin treatment over 2 days decreased the efficiency of gel contraction and, moreover, abolished the difference between PN-1−/− and wild-type DP cells (Fig. 4C). The involvement of thrombin in the stimulation of cell proliferation and gel contraction was quite surprising since its receptor, PAR-1, was not detected in vivo in the DP cells of anagen follicles from which our cultures were derived (Anan et al., 2003). PAR-1 was, however, homogeneously expressed in our DP cultures (Fig. 2C). The involvement of PAR-1 in the thrombin effect was substantiated because its activation by the thrombin receptor-activating peptide (TRAP) efficiently substituted for serum in the collagen gel contraction assay. A control peptide with the retro-sequence had no effect (Fig. 4D).

Thrombin stimulates both growth and contractibility through activation of the PI3K-Akt pathway

It is well documented that the PI3K pathway regulates cell proliferation by controlling protein synthesis and G1-S transition in different cell types (Collado et al., 2000; Medema et al., 2000). In addition, mTOR (also known as FRAP1) responds to growth factors independently or dependently of the PI3K pathway (Wullschleger et al., 2006). Consequently, specific inhibitors of PI3K and mTOR (LY294002 and RAD001, respectively) were added for 24 hours to test the involvement of these pathways in regulating cell cycle progression and change in cell volume. LY294002 had a strong effect on proliferation and volume increase of DP cells in culture (Fig. 5A,B). Furthermore, only LY294002
specifically abolished the difference between PN-1+/− and wild-type cells. RAD001 effectively inhibited volume increase and proliferation of DP cells but its effect was weaker and it did not eliminate the difference between the two cell types (Fig. 5A). Those effects were not biased by cytotoxicity, since no fragmented nuclei were observed. Moreover, aphidicolin, LY294002 and RAD001 showed no effect on cell viability, visualized by Trypan Blue staining. In each case, around 95% of the cells of each genotype were healthy at the end of the experiments when performed in 10% FCS supplemented medium (data not shown).

PI3K, as well as protein kinase C (PKC), also influenced the contractile properties of DP cells (Fig. 5C). Treatments with LY294002 or with a specific inhibitor of PKC (GF103203X) strongly limited collagen gel retraction. Nevertheless, only PI3K was required for thrombin-mediated stimulation of contraction. Indeed, after a pretreatment with hirudin, which abolished the difference between wild-type and PN-1+/− cells (Fig. 4C), addition of thrombin restimulated DP cell-mediated gel contraction, restoring the difference between the two cell types (Fig. 5C). This restimulation was prevented by LY294002 but insensitive to GF103203X (Fig. 5C). Thrombin stimulated contraction even in the presence of aphidicolin, indicating that its effect was not due to a differential increase in the number of wild-type and PN-1+/− cells (Fig. 5C).

Akt, as a downstream target of PI3K, was overactivated in the absence of PN-1. Whereas the total amount of Akt protein was similar in wild-type and PN-1+/− DP cells, the relative amount of phosphorylated protein was higher in PN-1+/− cells (Fig. 5D). This enhanced phosphorylation disappeared after a 24-hour treatment with LY294002, indicating that it was due to an increase in PI3K activity (Fig. 5D). As expected, addition of thrombin to wild-type DP cells led to a strong and rapid increase in Akt phosphorylation, which was also prevented by addition of LY294002 (Fig. 5E). PAR-1 was found to mediate the thrombin effect on the PI3K pathway. Indeed, TRAP but not the control peptide triggered Akt phosphorylation as efficiently as thrombin (Fig. 5F). Finally, the high level of Akt phosphorylation, as a result of the reduced inhibition of the serum-derived thrombin, was close to its maximum in PN-1+/− cells. Consequently, compared to wild-type cells, one-tenth the amount of purified thrombin was sufficient for Akt phosphorylation to reach a plateau in PN-1+/− cells (Fig. 5G).

The physiological relevance of PI3K pathway dysregulation was confirmed by comparing Akt phosphorylation in anagen whisker follicles from PN-1+/− and wild-type mice (Fig. 5H). By contrast to Akt, PTEN phosphorylation was not affected, indicating that this most important intracellular modulator of the PI3K pathway was not involved (Fig. 5H). The specificity of a PI3K pathway stimulation was further demonstrated by the observation that the Erk pathway was not affected by the absence of PN-1 (Fig. 5H). Finally, the PI3K signaling pathway was globally more active in anagen follicles of PN-1+/− mice, presumably as a consequence of Akt activation, because several downstream members of the PI3K signaling cascade, S6 kinase, GSK3 (glycogen synthase kinase 3) and members of the Forkhead box transcription factor FOXO family (FKHR and AFX), were also more phosphorylated (Fig. 5H).

Thrombin effect on cultured DP cells mimics changes occurring in vivo during catagen

Signaling from the DP during anagen stimulates growth and survival of neighboring keratinocytes (Botchkarev and Paus, 2003). We controlled that its function was maintained in vitro. We found...
Fig. 4. PN-1 protects DP cells from thrombin-stimulated expression of a myofibroblastic phenotype. (A) Level of SMA protein in cultured wild-type and PN-1+/− DP cell lysates (upper panel) and in extracts from the hairy skin of wild-type and PN-1−/− mice (unplucked; unP) and excised 3 days after stimulation of SMA expression by hair plucking (P) (lower panel). (B) Collagen gel contraction by embedded wild-type and PN-1+/− DP cells after 48 hours in medium containing 10% FCS. ***Significant difference from wild-type cells (P<0.001). (C) Effect of thrombin inhibition by hirudin (100 μM) on gel contraction by DP cells. (D) Effect of PAR-1-activating peptide (TRAP; 600 μM) or retro-sequence control peptide (600 μM) on the maintenance of contractile properties of wild-type DP cells transferred to a medium containing 1% serum. **Significant difference from 1% FCS (P<0.01).

Discussion

Our results provide evidence that thrombin stimulation of the PI3K-Akt pathway allows DP cells to express a DS-like phenotype. Because of the presence of thrombin, cultured whiskers DP cells accelerate proliferation, acquire new contractile properties, and simultaneously reduce their growth-supporting activity on keratinocytes. These effects require proteolytic activation of the thrombin receptor PAR-1 and are negatively controlled by the serine protease inhibitor PN-1. In vivo, myofibroblastic contractile properties are restricted to PAR-1-expressing DS cells during anagen and are enhanced in PN-1−/− follicles. Myofibroblastic characteristics are also acquired by wild-type pelage DP cells that drop their supporting activity on surrounding epithelial cells during catagen because they re-express PAR-1 and downregulate PN-1. Thus, our data strongly suggest that a tight control of thrombin proteolytic activity is one of the mechanisms of DP-DS cell plasticity.

Expression of thrombin coincides with that of PN-1. Furthermore, higher thrombin activity is detected in whiskers from mice lacking PN-1. These in vivo observations justified the use of PN-1−/− mice and of their DP cells to investigate the physiological effect of thrombin signaling. In vitro, determination of cell population doubling time, rate of BrdU incorporation and volume increase before mitosis provide evidence for the impact of PN-1 sensitive thrombin activity on DP cell proliferation. Of special interest is the observation that all BrdU-positive cells express the myofibroblastic marker SMA, indicating modifications in their contractile properties. This is confirmed by the enhanced contractile capabilities of the faster proliferating DP cells derived from PN-1−/− mice. Our results clearly indicate that the cellular events triggered by thrombin in highly specialized DP cells solely require activation of the PI3K-Akt pathway. Thrombin has also a dual effect on fibroblasts, namely stimulating both their proliferation (Chambard et al., 1987) and their differentiation into myofibroblasts (Bogatkevich et al., 2001). However, as these events are mutually exclusive (Grotendorst et al., 2004), the two effects of thrombin may be antagonistic. In these cells, stimulation of proliferation is mediated by the PI3K-Akt

that DP cells used as a feeder layer were indeed more efficient than DS cells in supporting keratinocytes growth (supplementary material Fig. S1C). We, therefore, compared the abilities of wild-type and PN-1+/− DP cells to sustain keratinocyte proliferation and survival in culture. The growth of human YF29 keratinocytes plated on confluent layers of inactivated DP cells was monitored (Fig. 6A) and quantified (Fig. 6B) after 8 days in culture. Keratinocytes plated on PN-1+/− DP cells formed threefold fewer clusters, which were much smaller than those formed on wild-type cells. PN-1-deficient DP cells supported the growth of 8- to 50-cell clusters, few having more than 30 cells. By contrast, on wild-type DP feeder layers, about 30% of the clusters contained 50-200 cells. The number of such large clusters was almost equal to the total number of clusters obtained on the PN-1+/− feeder layer.

As the keratinocyte culture medium also contained FCS, the results could reflect a protective effect of PN-1 against thrombin acting directly on keratinocytes, or a prior thrombin-dependent shift in DP cells properties. Therefore, we tested the effect of signaling molecules secreted by DP cells on keratinocytes plated on collagen and cultured in serum-free medium. As minimal medium conditioned by wild-type and PN-1−/− DP cells did not allow long term proliferation and survival of those cells, we evaluated the short term stimulation of the Akt pathway, known to sustain keratinocytes survival (Calautti et al., 2005), by monitoring the level of phosphorylated Akt by immunoblot analysis. As shown in Fig. 6C, medium conditioned by wild-type DP cells stimulated Akt phosphorylation in keratinocytes. This was abolished by pretreatment of DP cells with thrombin. Correspondingly, medium conditioned by PN-1−/− DP cells did not stimulate Akt phosphorylation (Fig. 6C). Thus, the reduced survival of keratinocytes on PN-1−/− DP feeder cells was most likely to be due to a direct effect of thrombin on DP cells leading to the loss of their growth-supporting activity.

In vivo, a shift in DP activity allows cell death and regression of the follicle during catagen (Botchkarev and Kishimato, 2003). Since PAR-1 upregulation and disappearance of PN-1 both occur in DP cells during catagen, we investigated whether the transformation seen in cultured PN-1−/− DP cells mimics the process taking place in vivo during catagen. As suspected, an increase in SMA was detected in protein extracts from catagen wild-type whisker follicles (Fig. 7A). Moreover, SMA protein was present only in the DS during anagen (Fig. 7B) but was strongly expressed in DP cells and in embedding trailer connective tissue during catagen (Fig. 7B.C). Thus, DP cells switched to myofibroblastic cells during catagen. Furthermore, catagen DP cells still expressed alkaline phosphatase (Fig. 7C). They thus exhibited markers of both DP and DS cells as observed for cultured DP cells (Fig. 2).

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pathway (Phillips-Mason, 2000) but differentiation requires PKC activation (Bogatkevich et al., 2001). Thus, in contrast to differentiating fibroblasts, myofibroblastic transition in DP cells does not require a distinct pathway and therefore seems to correlate with reversion to a more proliferative state restricted in vivo to the dermal sheath.

The simultaneous thrombin-induced loss of DP cell support of the growth and survival of keratinocytes argues for a shift in differentiation status rather than for a simple acquisition of new contractile properties. In fact, thrombin signaling on DP cells leads to cells expressing markers of both DP (alkaline phosphatase) and DS (smooth muscle actin) cells, therefore resembling DSC cells. Inductive properties (McElwee et al., 2003) and location of DSC cells as well as our in vitro data suggest that those cells are immediate precursors of DP cells. They may also be bipotent progenitors of both DP and DS cells. Whether or not thrombin-mediated stimulation of the PI3K-Akt pathway regulates dedifferentiation in hair follicle dermal cells is clearly an important question with both fundamental and practical implications.

The action of thrombin on DP cells requires the presence of its receptor PAR-1, because it can be mimicked by addition of synthetic thrombin-receptor-activating peptide. The absence of PAR-1 in DP cells during anagen, seems to require epithelial-mesenchymal interactions because isolated cells spontaneously re-express the receptor in culture. The reverse of this, anagen-specific expression of a versican-GFP transgene rapidly disappears in cultured DP cells as it does in vivo during catagen (Kishimoto et al., 1999). Kishimoto et al. previously proposed that the anagen-specific pattern of gene expression in DP cells exhibits a spontaneous procatagen drift in culture. However, our data show that in vitro drift actually correlates with the acquisition of a DS-like phenotype by DP cells. This predicts that genes active in both dermal cell types would not switch.

Fig. 5. Thrombin stimulates both growth and contractibility through activation of the PI3K/Akt pathway. (A) Percentages of wild-type and PN-1−/− (KO) cells incorporating BrdU (10 μM, 2 hours). The PI3K inhibitor LY294002 (LY; 50 μM) and the mTOR inhibitor RAD001 (RAD; 20 nM) were added in 10% FCS medium 24 hours before BrdU incorporation. **Significant difference from wild-type cells (P<0.001). (B) Volume increase of cells switched to 1% FCS medium and arrested with aphidicolin (1 μg/ml, as in Fig. 3) determined after 24 hours with or without LY294002 or RAD001. *Significant difference from wild-type cells (P<0.05). (C) Effects of LY294002 or GF103203X (5 μM) on thrombin-stimulated (1 U/ml) collagen gel contraction by wild-type and PN-1−/− (KO) DP cells pretreated with hirudin (100 μM, 6 hours) and cultured in the presence of hirudin and aphidicolin. **Significant difference from wild-type cells (P<0.05). (D) Relative levels of phospho-Akt and total Akt proteins in extracts from wild-type and PN-1−/− DP cells assessed by immunoblotting with the indicated antibodies. Cells were either untreated (/) or cultured in the presence of LY294002 or RAD001 as in A. (E) Effect of thrombin on Akt phosphorylation examined by adding thrombin (1 U/ml, 30 minutes) to wild-type DP cells grown in medium containing 1% FCS. The dependence of the thrombin effect on PI3K pathway was assayed by adding LY294002 or RAD001 1 hour prior to thrombin. (F) Involvement of PAR-1 in Akt activation. Wild-type DP cells were treated with thrombin (as in E), TRAP or control peptides (as in Fig. 4). (G) Comparison of the efficiency of thrombin stimulation of Akt phosphorylation in wild-type versus PN-1−/− DP cells tested as in D. (H) Phosphorylation status of proteins of the PI3K pathway in lysates from dissected anagen whisker follicles of 20-day-old wild-type and PN-1−/− littermates.
expression in vitro. Although this hypothesis needs further confirmation, it is worth noting that expression of PN-1 is indeed maintained in isolated cultured DP cells.

The upregulation of PAR-1 and the downregulation of PN-1 during catagen seem to indicate a double security system to optimally support the efficiency of thrombin signaling in this phase. Furthermore, the differential expression of PAR-1 in DP and DS during anagen and its upregulation in DP during catagen leads to the intriguing question of whether PAR-1 expression in DS is needed in vivo for the acquisition or maintenance of a non-DP phenotype. It is likely that distinct regulation of PAR-1 expression in DP and DS depends upon their different location and the different epithelial cell types with which they interact. Interestingly, combining DS cells with hair matrix cells can confer the capacity to act as DP-like cells when grafted (Reynolds and Jahoda, 1996). Analysis of how the cross-talk between matrix and papilla cells influences PAR-1 expression, and thus the function of the thrombin regulatory network, might improve our understanding of the regenerative properties of hair follicles.

DP cells can be implanted in adult skin, where they induce the formation of new hair follicles (Jahoda and Reynolds, 1996). However, cultured DP cells progressively lose their inductive capacity upon passages (Jahoda et al., 1984; Kishimoto et al., 1999). They can, however, induce new follicles when implanted together with hair matrix cells (Reynolds and Jahoda, 1996). Alternatively, the loss of inductive capacity can be prevented by co-culture with Wnt3a-producing feeder cells (Kishimoto et al., 2000). In this respect, our data support a previous observation of Yu et al. (Yu et al., 1995), who suggested a positive correlation between the relative level of PN-1 expression in immortalized DP cell lines and their ability to support hair growth in an in vivo follicular reconstitution assay. This argues for a primordial role of thrombin-induced DP to DS-like shift in loss of inductive capacity in vitro. Thus, our findings could be relevant for skin and hair follicle reconstitution in providing new tools for the in vitro engineering of hair follicle dermal cells. For example, in vitro drift might be largely prevented or reverted upon exposure to inhibitors of the thrombin-PAR-1-PI3K pathway.

Our results also stress the importance of the difference in control of PAR-1 and PN-1 expression for regulating myofibroblastic properties of dermal cells during the follicular cycle. In the DS, the presence of PAR-1, thrombin and PN-1 during anagen suggests a fine-tuning of DS cell properties sensitive to thrombin. Accordingly, enhanced expression of SMA contractile protein and overactivation of the PI3K-Akt pathway are observed in anagen hair follicles of PN-1−/− mice. In the DP, by contrast, the downregulation of PAR-1, the presence of PN-1 and the efficient elimination of PN-1-thrombin complexes prevent thrombin signaling during anagen. This observation could have a more general biological relevance. It was shown that thrombin causes a marked delay in myogenesis (Guttridge et al., 1997) and that PAR-1 signaling is repressed upon myocytes fusion (Suidan et al., 1996). Using a myoblastic cell line, we found that a drastic PAR-1 downregulation and strong PN-1 upregulation are simultaneously detected upon myotube formation (supplementary material Fig. S2). This could indicate a more general significance of this double-lock principle. In hair follicles, the need for such a drastic...
lock is clearly supported by our results suggesting that DP cells would otherwise lose their ability to support keratinocytes. Lack of PN-1 in knockout mice is not sufficient to shift the DP cell phenotype during anagen. However, in wild-type, the simultaneous disappearance of PN-1 (Yu et al., 1995) and the re-expression of PAR-1 (Anan et al., 2003) during catagen correlates with de novo expression of SMA. Functionally, this enhancement of contractile properties by dermal cells during regression of the follicle makes sense because it favors maintenance of tight contacts between disorganized tissues and possibly helps the upward movement of the follicle.

Change in size and cross-regeneration of dermal tissues involve coordinated control of proliferation and migration of cells between compartments (Jahoda, 1992; Tobin et al., 2003; McElwee et al., 2003). Furthermore, renewal of dermal cells is coupled with hair follicle cycling (Tobin et al., 2003). Since thrombin stimulates hair follicle dermal cell proliferation in vitro, future studies may show if in vivo cyclic up and down expression of thrombin regulators helps such coupling. As thrombin activation of the PI3K-Akt pathway simultaneously induces myofibroblastic conversion, cell renewal may also rely on reversibility of differentiation status.

Materials and Methods

Animals

PN-1−/− (IacZ/iacZPN-1−/−) (PN-1 KI) mice (Kvajo et al., 2004) and PN-1+/+ mice (Luthi et al., 1997) were each backcrossed for 12 generations into the C57BL/6 line (Arbresles, France). All animal experiments were approved by the Swiss veterinary authorities.

X-gal staining, immunohistochemistry and immunocytochemistry

Vibriossal follicles were dissected out, fixed in Bouin’s solution or 4% paraformaldehyde (PFA), and embedded in paraffin or in OCT (Sakura Finetek). For β-galactosidase histochemistry, longitudinal sections were stained according to standard procedures (Sanes et al., 1986) and counterstained with Nuclear Fast Red (Vector Laboratories).

Longitudinal sections (8-12 μm thick) were incubated with various antibodies: rabbit anti-thrombin (1/200, American Diagnostica), anti-human smooth muscle actin/HRP (1/200, DakoCytomation), mouse anti-PN-1 (1/48, 1/200 (Manusy et al., 1993)) and stained with the Vectastain ABC peroxidase system (Vector Laboratories).

For immunocytochemical staining, cells were incubated with mouse anti-PN-1 (1/200), rabbit anti-LRP (1/200); a generous gift from D.K. Strickland, University of Maryland School of Medicine, Baltimore, MD), mouse anti-BrdU (1/300; BD Pharmingen), anti-alpha-smooth muscle actin (1/200, Sigma-Aldrich) and anti-NCAM NCL-L-CD56-1B6 (1/200, DakoCytomation), PAR-1 (1/1000; Immunotech) and PN-1 (4B3 1/1000). Signals for phospho-GSK3(Ser9), phospho-Akt(Ser473), phospho-S6 kinase 1(Thr389), phospho-FKHR(Ser256), phospho-ERK (Thr202/Tyr204), phospho-P70-S6 kinase and phospho-p70-S6 kinase were visualized with DAB.

Cell culture

Anagen vibriossal follicles from 3-month-old PN-1 wild-type (PN−/−) and PN−/− mice were microdissected (Kobayashi et al., 1993) and the dermal papillas were mechanically removed and dissociated in 1× trypsin-EDTA (Gibco-BRL). Rat DS cells were obtained as described previously (Jahoda et al., 1991). Dissociated cells were cultured in DMEM containing 10% FCS and 20 ng/ml human EGF transferred to a 24-well plate (300 μl/well; Nunc) and incubated at 37°C under 10% CO2 for 30 minutes. DMEM (500 μl) containing 10% FCS, 20 ng/ml EGF and 1 μg/ml of aphidicolin was layered over the gel. Several experiments were carried out in the presence of hirudin (100 μM), LY294002 (50 μM), GF109203X (5 μM; Biomol), thrombin-receptor-activated peptide (TRAP; 600 μM; SFLRAMide, Bachem) or retro-sequence peptide (600 μM control, retro-SLIGV, Bachem). Contraction data are presented as the percentage reduction in gel surface.

Keratinocyte survival assay

Human epidermal keratinocytes, strain YF29, were cultivated as previously described by Rheinwald and Green (Rheinwald and Green, 1975). They were then seeded (15,000 cells/cm2) on a feeder layer of mitomycin C (10 μg/ml, 2 hours; Sigma-Aldrich)-treated wild-type, PN−/− DP cells or rat DP, DS cells and cultured in cFAD keratinocyte medium ( Gibco-BRL). On day 8, cells were fixed and stained with 1% Rhodamine B (Fluka) as described previously (Brouard et al., 1999). Clusters from eight or more keratinocytes were counted in the sampled central areas. Experiments were done in triplicate.

To test DP cell signaling effects on the stimulation of the PI3K/Akt survival pathway in keratinocytes, DP-conditioned medium (DPCM) was applied to YF29 cells for 30 minutes. DPCM was obtained by incubating serum-free keratinocytes medium (SFKM, Gibco-BRL) for 24 hours with wild-type or PN−/− DP cell pretreated or not with recombinant thrombin (1 U/ml; Sigma-Aldrich).

Proteolytic assays

Anagen vibriossal follicles were dissected out and homogenized. Total protein concentration was determined (BioRad) and aliquots (10 μg) of homogenates were mixed with S-2238 (H-D-Phe-Pip-Arg-pNA-2HCl) substrate (1.25 mg/ml, Chromogenix) and the amidolytic activity immediately determined as described previously (Hengst et al., 2001).

Immunoblot analysis

SDS-PAGE and immunoblot analyses were used to identify phospho-PTEN(Ser380), phospho-Akt(Ser473), phospho-26 kinase 1(Thr389), phospho-FKHR(Ser256), phospho-GSK3Ser9, (all antibodies at 1/1000; Cell Signaling), HRP-SMA (1/8000; DakoCytomation), PAR-1 (1/1000; ImmunoTech) and PN−/− (1/48) 1/1000). Signals were compared with those obtained with related antibodies (for p70-S6 kinase, and Akt, 1/1000; Cell Signaling), and/or with anti-actin (1/3000; NeoMarkers).

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References


