Increased keratinocyte proliferation by JUN-dependent expression of PTN and SDF-1 in fibroblasts

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Summary

In skin, fibroblasts of the connective tissue play a decisive role in epidermal homeostasis and repair by contributing to the regulation of keratinocyte proliferation and differentiation. The AP-1 transcription factor subunit JUN plays a crucial role in this mesenchymal-epithelial interplay by regulating the expression of two critical paracrine-acting cytokines, keratinocyte growth factor (KGF) and granulocyte-macrophage colony-stimulating factor (GM-CSF). We have performed gene expression profiling of wild-type and *Jun*$^{-/-}$ mouse embryonic fibroblasts to identify additional players involved in this complex network, and have found pleiotrophin (PTN) and the stromal cell-derived factor 1 (SDF-1) as novel JUN-regulated factors. Both cytokines are expressed by dermal fibroblasts in vivo, as shown by semi-quantitative RT-PCR and in situ hybridization on murine skin sections. Using a heterologous feeder layer co-culture system, we demonstrated that PTN and SDF-1 exert a mitogenic effect on primary human keratinocytes. Moreover, SDF-1-induced keratinocyte proliferation could be specifically inhibited by neutralizing antibodies against SDF-1 or its receptor, CXCR4. Consistent with its role in promoting keratinocyte growth, PTN was upregulated during cutaneous wound healing in vivo. Interestingly, co-cultivation with keratinocytes stimulated PTN expression but repressed SDF-1 production in fibroblasts, demonstrating the complexity of the paracrine regulatory cytokine networks that control skin homeostasis and regeneration.

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Key words: AP-1, Skin, Mesenchyme, SDF-1, CXCL12, Pleiotrophin, HB-GAM

Introduction

Interactions between mesenchymal and epithelial cells play an important role in regulating tissue development, homeostasis and regeneration. This is well documented in skin, where a cytokine network established by a mutual crosstalk between mesenchymal and epithelial cells is crucially involved in the tight control of keratinocyte proliferation and differentiation (Tuan et al., 1994; Maas-Szabowski et al., 1999). These interactions via diffusible factors are of vital importance during cutaneous wound healing, when enhanced keratinocyte proliferation is required for rapid re-epithelialization (Martin, 1997; Singer and Clark, 1999; Werner and Grose, 2003).

The crucial role of the AP-1 transcription factor subunit JUN in the trans-regulatory control of keratinocyte proliferation was first shown in an in vitro heterologous organotypic skin equivalent composed of murine fibroblasts and primary human keratinocytes, in which wild-type fibroblasts allowed keratinocytes to proliferate and to build up a well stratified epithelium resembling normal human skin (Szabowski et al., 2000). In the presence of *Jun*-deficient fibroblasts, keratinocyte proliferation was dramatically reduced, and only a poor epithelium was formed. This phenotype could be attributed in part to the loss of JUN-dependent transcriptional activation of the genes encoding keratinocyte growth factor (*Kgf*; also known as fibroblast growth factor 7, *Fgf7*) and granulocyte-macrophage colony-stimulating factor [*Gm-csf*; also known as colony-stimulating factor 2 (granulocyte-macrophage), *Csf2*] in mutant fibroblasts. Cell-autonomous JUN activity in keratinocytes regulates expression of autocrine factors and their receptors, such as HBEGF and EGFR, respectively. However, these factors seemed not to be absolutely required for skin development and homeostasis, as genetically modified mice lacking JUN specifically in epidermal keratinocytes did not display an obvious skin phenotype (Li et al., 2003; Zenz et al., 2003). Moreover, the proliferation defect of cultured keratinocytes isolated from these mice could be rescued in vitro by the addition of recombinant HBEGF as well as by the paracrine acting factors keratinocyte growth factor (KGF) and GM-CSF (Zenz et al., 2003), supporting the importance of diffusible factors for keratinocyte growth. Moreover, these results suggested that in vivo the cell-autonomous proliferation defect of *Jun*-deficient keratinocytes might be functionally compensated for by mesenchyme-derived growth factors.

Here, we report on two novel JUN-activated cytokines in

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fibroblasts, the heparin-binding factor pleiotrophin (PTN; also known as heparin-binding growth-associated molecule, HB-GAM) and the stromal cell-derived factor-1 [SDF-1; also known as chemokine (C-X-C motif) ligand 12, CXCL12], which we identified in a microarray screen designed to elucidate the diversity of JUN-dependent genes involved in mesenchymal-epithelial interactions during cutaneous wound repair (Florin et al., 2004). We determined the expression of both factors in murine skin in vivo during tissue homeostasis and wound healing and analysed their functional impact on keratinocyte growth in vitro.

Materials and Methods

Cell lines and cultivation

Normal human epidermal keratinocytes (NEKs) were derived from adult skin obtained from surgery as previously described (Smola et al., 1993; Stark et al., 1999). NEKs were expanded on X-irradiated feeder cells (5000 cells/cm^2) in FAD medium (DMEM:Ham's F12, 3:1) with 100 U/ml penicillin, 50 μg/ml streptomycin and supplemented with 5% foetal calf serum (FCS), 5 μg/ml insulin, 1 ng/ml recombinant human EGF, 10^{-10} M cholera toxin, 10^{-4} M adenine and 0.4 μg/ml hydrocortisone (Sigma, Deisenhofen, Germany) as described previously (Smola et al., 1993).

Immortalized wild-type and *Jun*-deficient mouse embryonic fibroblasts (MEFs) (Schreiber et al., 1999; Andrecht et al., 2002) were grown in DMEM (Bio Whittaker, Serva, Heidelberg, Germany) supplemented with 10% FCS. For feeder layer co-cultures, trypsinized fibroblast suspensions (at 5×10^5 cells/ml) were γ-irradiated with 15 cGray and plated at 3.5×10^3/cm^2 in DMEM either on plastic dishes (for phase contrast microscopy) or glass slides (for immunofluorescence analyses). After attachment of the fibroblasts, NEKs were plated onto the feeder cells (10.5×10^3/cm^2) in FAD. Thereafter, medium was replaced by fresh supplemented FAD every 48 hours without additives (controls) or with any of the following recombinant human factors: 25 ng/ml PTN, 50 ng/ml SDF-1, 10 ng/ml KGF, 5 μg/ml anti-CXCR4 and 10 μg/ml anti-SDF-1 (all R&D Systems, Wiesbaden, Germany).

Indirect immunofluorescence microscopy

Glass slides of 6-day-old co-cultures were fixed for 5 minutes in 80% methanol at 4°C followed by 2 minutes in acetone at –20°C, rehydrated in PBS and blocked for 30 minutes in 1% BSA/PBS. Antibodies were then applied in 1% BSA/PBS for 3 hours at room temperature in a moist chamber. After washing in PBS, slides were incubated for 1 hour with species-specific, fluorochrome-conjugated secondary antibodies (Dianova, Hamburg, Germany) as well as 0.5 μg/ml bisbenzimide dye (Hoechst No. 33258) for nuclear counterstaining. Finally, the slides were mounted in Mowiol (Medim, Gießen, Germany).

Keratinocyte differentiation was visualized by staining for mouse anti-human keratin1/10 (clone 8.60; Sigma) and involucrin (clone I9018; Sigma). Proliferating cells were labelled by rabbit anti-human MIB1 (Clone M7240, Dako, Hamburg, Germany) and quantified by counting mindbomb homolog 1 (MIB1)-positive cells with respect to the total number of keratinocytes per island.

RNA isolation and RT-PCR

For gene expression profiling, logarithmically growing wild-type and *Jun*-deficient MEFs were starved by culturing in medium containing 0.5% FBS for 48 hours and afterwards either left untreated or stimulated with 5 ng/ml interleukin-1 alpha (R&D Biosystems, GmbH, Wiesbaden, Germany) for 7 hours. To analyse RNA levels in murine skin, dermis and epidermis were separated after thermolysine treatment (Sigma, Deisenhofen, Germany) for 1-2 hours at room temperature, frozen in liquid nitrogen and stored at –80°C. Total RNA was isolated using a protocol combining TRIzol® reagent (Invitrogen GmbH, Karlsruhe, Germany) and subsequent column purification (RNeasy Kit, Quiagen, Hilden, Germany). RNA integrity was controlled by gel electrophoresis; the quantity was determined photometrically. cDNA synthesis was performed according to standard protocols using oligo(dT)- and random hexamer-oligonucleotides. For semi-quantitative RT-PCR, gene-specific fragments were obtained by linear phase PCR amplification, standardized for β-tubulin 1 (*Tubb1*) or hypoxanthine guanine phosphoribosyl transferase 1 (*Hprt1*) levels. Quantitative differences in mRNA levels during wound repair were determined by real-time RT-PCR using the Absolute™ QPCR SYBR® Green Fluorescein Mix (ABgene, Surrey, UK) and a thermal cycler controlled by the MyiQ Real Time Detection System software (BioRad, Munich, Germany).

Wound healing experiments

Four round full-thickness excisional wounds (4 mm diameter) were generated on the back of female BALB/c mice using fine scissors. The wounds were left untreated. For RNA expression studies, complete wounds including 2 mm of the epidermal margins were excised at different time points after injury, immediately frozen in liquid nitrogen, and stored at –80°C until used for RNA isolation. Wound healing experiments were performed with permission from the local authorities in Zurich, Switzerland.

In situ hybridization

In situ hybridization was performed on 7 μm cryosections of mouse back skin. Sense and anti-sense probes were generated by run-off in vitro transcription of the corresponding PCR fragments cloned into the vector pGEM-T-Easy (Promega GmbH, Mannheim, Germany) in the presence of digoxigenin (DIG)-labelled dUTPs. The hybridization was performed at 55°C for 4 hours. Hybridized probe was detected by peroxidase-conjugated anti-DIG antibodies (Roche Diagnostics) using diaminobenzidine (DAB; Vector Laboratories Ltd., Peterborough, UK) as substrate. Sections were counterstained with Haematoxylin.

Results

Expression of pleiotrophin and SDF-1 in fibroblasts is JUN dependent

In a recent microarray experiment designed to identify AP-1 target genes in fibroblasts (Florin et al., 2004), we found two cytokines, pleiotrophin and SDF-1, to be activated by the AP-1 transcription factor subunit JUN (Fig. 1). Gene expression profiling revealed strongly reduced expression levels of both cytokines in $Jun^{-/-}$ cells when compared to wild-type fibroblasts, indicating that JUN activity is required for the expression of *Ptn* and *SDF-1* in these cells. Similar results were obtained for *Kgf*, a known JUN-activated gene (Szabowski et al., 2000). In comparison, the disabled homolog 2 (*Dab2*) gene, which was recently identified to be repressed by JUN (Florin et al., 2004), was expressed at higher levels in the deficient cells, whereas the JUNB target genes lipocalin-2 (*Lcn2*) and CD24a antigen (*Cd24a*) (Florin et al., 2004) did not show significant differences in expression levels between wild-type and $Jun^{-/-}$ cells (Fig. 1A). Accordingly, when performing semi-quantitative RT-PCR on mRNA isolated from untreated or IL-1-stimulated fibroblasts, *Ptn* and *Sdf-1* were found to be expressed by wild-type cells, whereas no specific transcripts

Journal of Cell Science

could be detected in cells lacking JUN (Fig. 1B). IL-1 treatment of the fibroblasts had no effect on PTN or SDF-1 production, whereas mRNA levels of the SDF-1 receptor CXCR4, KGF and lipocalin-2 were confirmed to be upregulated following IL-1 stimulation as described (Brauchle et al., 1994; Chedid et al., 1994; Gupta et al., 1998; Florin et al., 2004).

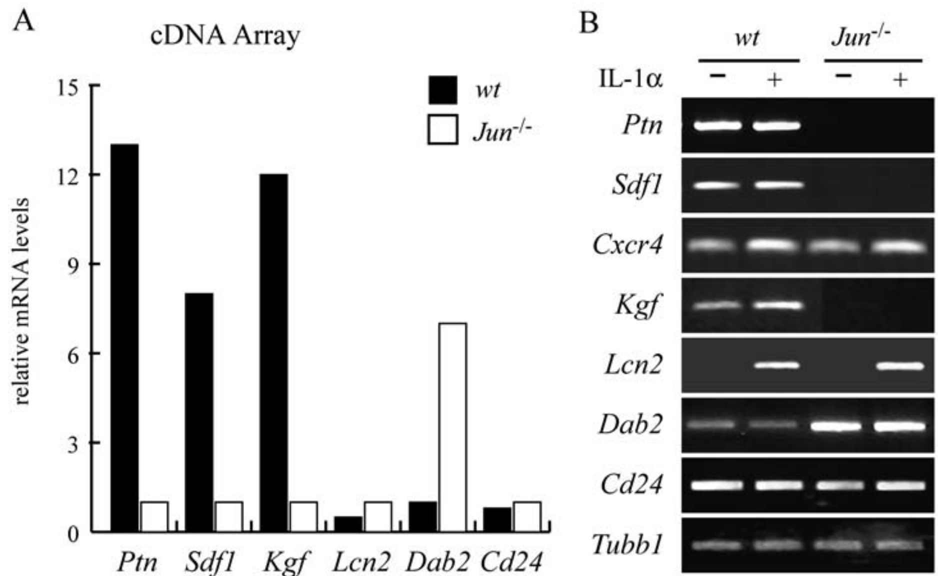


Fig. 1. Expression of PTN and SDF-1 is JUN dependent. (A) Expression profiling of wild-type versus *Jun*$^{-/-}$ mouse embryonic fibroblasts revealed *Ptn* and *Sdf-1* to be activated by JUN. Enhanced transcript levels in wild-type cells (black bars) are indicated as fold difference compared to the expression in *Jun*$^{-/-}$ cells (white bars), which was set as 1. *Kgf*, representing a known direct JUN target, the JUN-repressed disabled homolog 2 (*Dab2*) and the JUN-independent genes lipocalin (*Lcn2)* and CD24a antigen (*cd24a*) were included as controls. (B) Validation of the microarray results by semi-quantitative RT-PCR on cDNA derived from untreated and IL-1-stimulated fibroblast monocultures. Expression of the JUN-activated cytokines *Ptn*, *Sdf-1* and *Kgf* was impaired in fibroblasts lacking JUN activity, whereas *Cxcr4*, *Lcn2*, *Dab2* and *Cd24a* levels were not reduced. β-tubulin 1 (*Tubb1*) was used for standardization.

PTN and SDF-1 are expressed by dermal fibroblasts in vivo

To analyse the contribution of mesenchymal and epithelial cells to PTN and SDF-1 expression, dermis and epidermis from the back skin of mice were dissociated by thermolysine treatment, and semi-quantitative RT-PCR was performed using gene-specific oligonucleotide primers (Fig. 2A). The separation of the two compartments, as monitored by the expression of the keratinocyte differentiation marker keratin 10 (*K10*; also known as keratin complex 1, acidic, gene 10, *Krt1-10*) and the fibroblasts-specific gene procollagen type I, α2 (*Col1a2*), was almost complete, except for a weak keratin 10 signal in the dermal preparation resulting from the presence of some residual hair follicles. In agreement with the expression in fibroblasts in vitro (Fig. 1) and undetectable levels in cultured primary mouse keratinocytes (see supplementary material Fig. S1), mRNA transcripts of both cytokines, PTN and SDF-1, were predominantly detected in the dermal fraction, with PTN being also detectable, but at lower levels, in cells of the epidermis (Fig. 2A). The PCR data were further confirmed by in situ hybridization on skin sections (Fig. 2B), where dermal fibroblasts stained strongly for both cytokines. Thus, we identified *Ptn* and *Sdf-1* as two novel JUN target genes that are expressed by mesenchymal cells in the skin in vivo.

PTN and SDF-1 enhance keratinocyte proliferation

The crucial importance of the JUN-dependent growth factor supply for epithelial cell growth was demonstrated in heterologous co-culture systems composed of primary human keratinocytes cultivated together with genetically modified

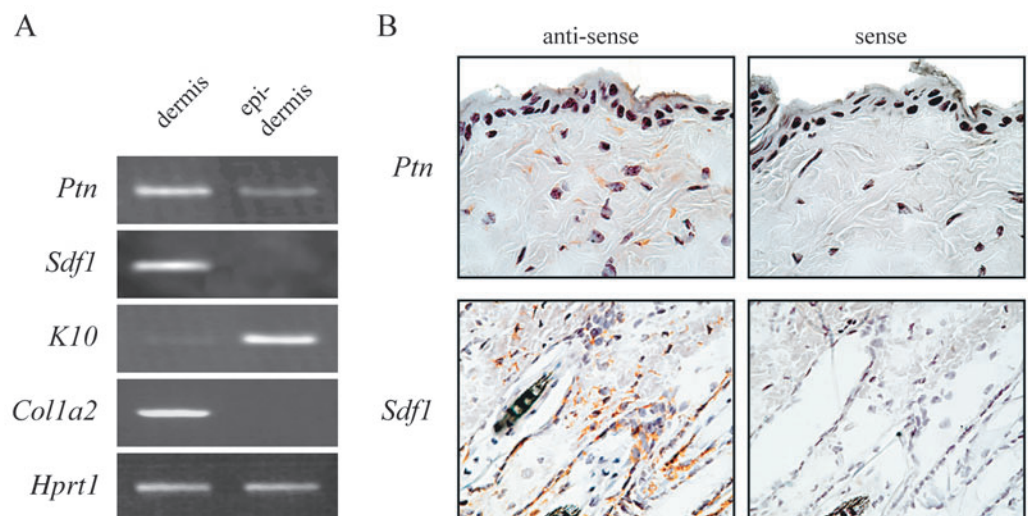
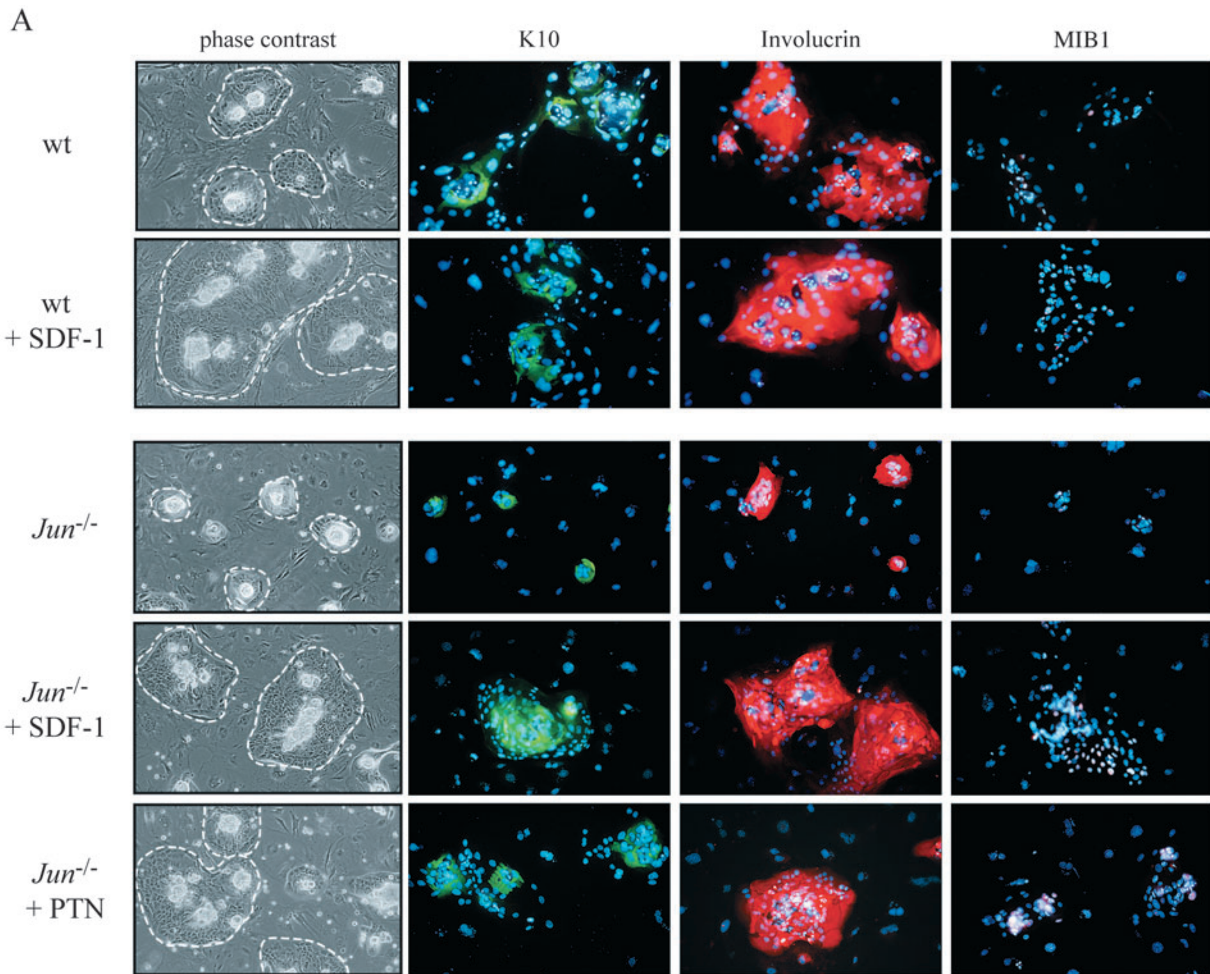


Fig. 2. PTN and SDF-1 are expressed by dermal fibroblasts in vivo. (A) Dermis and epidermis of mouse back skin were dissected and semi-quantitative RT-PCR was performed to reveal the expression of *Ptn* and *SDF-1* in the dermal compartment. Keratin 10 (*K10*) and procollagen type I, α2 (*Col1a2*) were used as controls for the separation. The cDNA was standardized to *Hprt* levels. (B) In situ hybridization using DIG-labelled riboprobes for *Ptn* and *Sdf-1* confirmed dermal fibroblasts as a source of cytokine expression. Hybridization was visualized using diaminobenzidine (DAB, reddish-brown signal) and nuclei were counterstained with Hematoxylin.



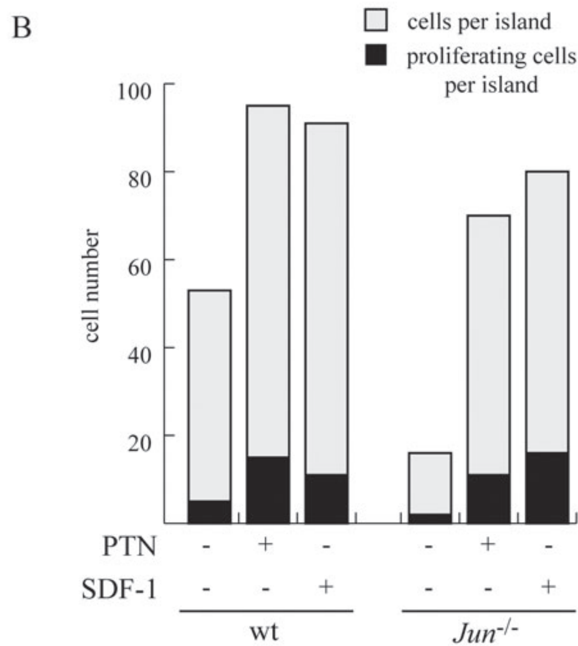


Fig. 3. SDF-1 and PTN stimulate proliferation of primary keratinocytes in vitro. (A) Heterologous feeder-layer co-cultures grown in the presence or absence of recombinant human SDF-1 (50 ng/ml) or PTN (25 ng/ml) were analysed by phase contrast microscopy (left column) and immunofluorescence (right columns). Individual keratinocyte islands are outlined with dashed lines in the phase contrast images. Differentiation and proliferation of primary keratinocytes was visualized by staining for keratin 10 (early differentiation), involucrin and MIB1 (proliferation). (B) Quantification of the mitogenic effect of PTN and SDF-1 on primary keratinocytes. MIB1-positive cells of at least 10 colonies were counted and plotted with the total number of cells per keratinocyte colony (shaded bars).

murine fibroblasts (Szabowski et al., 2000). To analyse whether the novel JUN-dependent cytokines PTN and SDF-1 also had an impact on keratinocytes, feeder-layer co-cultures containing wild-type or *Jun*$^{-/-}$ fibroblasts were either left untreated or supplemented with the respective recombinant human factor (Fig. 3). Keratinocytes seeded onto wild-type feeder cells grew

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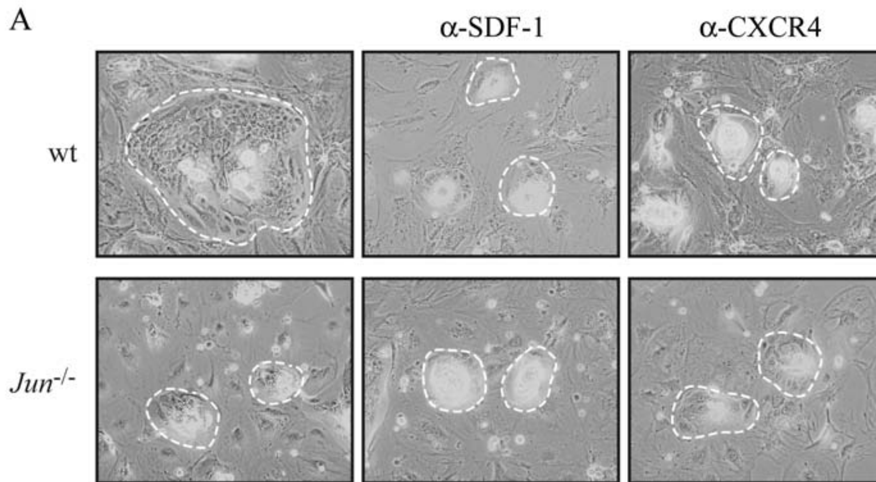

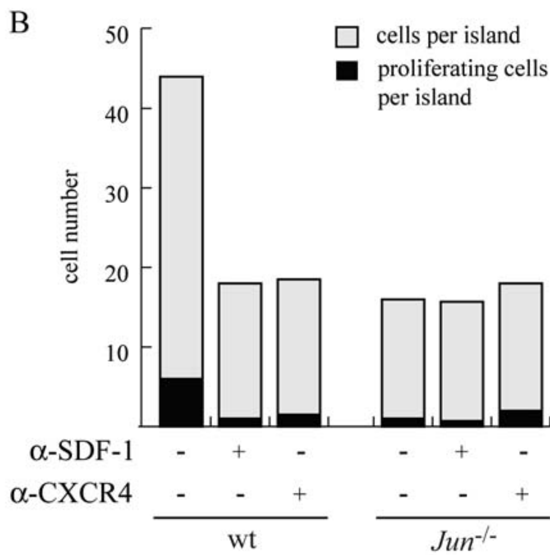

Fig. 4. Neutralization of SDF-1 activity impairs keratinocyte proliferation (A) Phase contrast microscopy demonstrating the inhibitory effect of neutralizing antibodies against either SDF-1 (10 μg/ml) or its receptor CXCR4 (5 μg/ml) on keratinocyte growth. Individual islands are outlined with dashed lines. (B) Proliferating cells and colony size were quantified as in Fig. 3B.

out to form cell islands with central bright areas in the phase contrast image indicating differentiated cells (Fig. 3A). In co-cultures containing *Jun*-deficient fibroblasts, the keratinocyte islands were strongly reduced in size, but the cells retained the ability to differentiate, as further analysed by immunofluorescent staining for the early differentiation markers keratin 10 and involucrin. When mitotic cells were labelled by MIB1 immunostaining, a clear reduction in keratinocyte proliferation was observed in co-cultures with *Jun*$^{-/-}$ fibroblasts compared to wild-type feeder cells.

Addition of recombinant PTN or SDF-1 to the medium of *Jun*-deficient co-cultures resulted in strongly enlarged keratinocyte colonies, as seen by phase contrast microscopy and involucrin immunostaining (Fig. 3A). Correspondingly, an enhanced proliferation of the keratinocytes was observed, as quantified by the number of MIB1-positive cells together with the total number of keratinocytes per colony (Fig. 3B). When the recombinant factors were added into cultures containing wild-type fibroblasts, keratinocyte proliferation and thus colony size were also increased, although this was less pronounced than in *Jun*$^{-/-}$ co-cultures. The proliferative impact of both cytokines was clearly dose dependent, as mild effects were visible when 10 ng/ml of recombinant factor were added, but best results were achieved with 25 ng/ml PTN and 50 ng/ml SDF-1 (data not shown).

The role of SDF-1 as a mitogen for primary keratinocytes was further demonstrated by interfering with SDF-1 signalling in wild-type co-cultures (Fig. 4). Application of neutralizing antibodies against either SDF-1 or its receptor CXCR4 led to a dramatic reduction in the size of keratinocyte colonies. The number of proliferating cells was also reduced to levels resembling those of untreated co-cultures with *Jun*$^{-/-}$ fibroblasts (data not shown). As expected, no further inhibition of keratinocyte growth was observed, when neutralizing antibodies were added to cultures containing *Jun*-deficient fibroblasts, which did not express *Sdf-1* at detectable levels (Fig. 1B).

Taken together, addition of both cytokines, PTN and SDF-1, rescued the growth inhibition of keratinocytes in the presence of *Jun*$^{-/-}$ feeder-layer fibroblasts, and this effect could be specifically attributed to the mitogenic activity of both factors on primary keratinocytes in this system.

Regulation of PTN and SDF-1 expression in wounds

Since the feeder-layer co-culture system is regarded as a model for the initial phase of cutaneous wound healing, we asked whether expression of PTN and SDF-1 would be affected during wound repair in order to support keratinocyte proliferation and thus accelerate re-epithelialization. By semi-quantitative and real-time PCR analysis, a two-fold induction of *Ptn* was detected 6 hours after injury (Fig. 5A,B), when mRNA levels of GRO-1, a neutrophil chemoattractant that is well known to be upregulated in wounds (Nanney et al., 1995), reached their maximum. *Kgf*, a known wound-regulated JUN target gene (Werner et al., 1992), was most prominently expressed at day 3 post-wounding (Fig. 5B). Interestingly, *Sdf-1* mRNA levels did not change significantly during the process of cutaneous repair (Fig. 5A,B).

Moreover, stimulation of fibroblast monocultures with the proinflammatory cytokine IL-1, as one important signal in skin wounds, had no impact on SDF-1 or PTN expression (Fig. 1B). However, PTN expression was upregulated when the fibroblasts were stimulated by the presence of co-cultivated

Journal of Cell Science

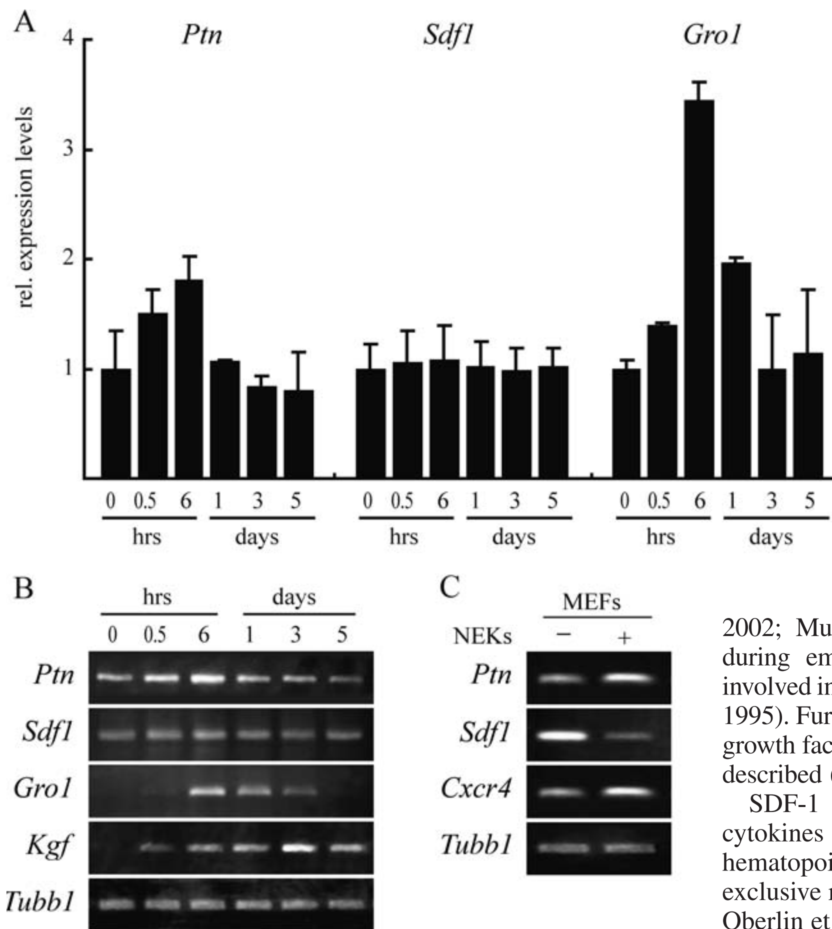

Fig. 5. Regulation of PTN and SDF-1 in wounds and co-cultured fibroblasts. (A) Real-time PCR and (B) semi-quantitative RT-PCR was performed on cDNA from full-thickness excisional mouse dorsal skin wounds prepared at the indicated times after injury. (A) Changes in mRNA levels during the wound healing process are plotted relative to the expression in unwounded skin (0 hours, set as 1). Average values of three independent measurements are shown. (B) Expression analysis on RNA from an independent wounding experiment. Essentially identical results were obtained in a third independent RNA analysis of expression kinetics during wound healing (data not shown). *Gro1* (A,B) and *Kgf* (B), both known to be induced by wounding, were included as positive controls. (C) Expression of *Ptn*, *Sdf-1* and *Cxcr4* in fibroblast monocultures or co-cultures with primary human normal epidermal keratinocytes (NEKs). *Ptn* and *Cxcr4* were upregulated, whereas *Sdf-1* transcript levels decreased in the presence of co-cultured keratinocytes.

keratinocytes (Fig. 5C), arguing for the involvement of at least one additional keratinocyte-derived factor, which induces gene expression in the feeder cells. Interestingly, *Sdf-1* was downregulated in fibroblasts upon co-cultivation with keratinocytes, whereas mRNA levels of its receptor *Cxcr4* increased under these conditions.

Discussion

Re-epithelialization and tissue homeostasis of the skin are co-ordinately regulated by cell-cell and cell-matrix interactions as well as autocrine and paracrine acting diffusible factors (Fuchs, 1990; Fusenig, 1994). In an in vitro skin equivalent, the trans-regulatory function of the transcription factor JUN, activating the expression of the paracrine growth factors KGF and GM-CSF, was shown to be required for normal keratinocyte proliferation (Szabowski et al., 2000). Here, we report on the identification of two novel JUN activated cytokines, PTN and SDF-1, which are expressed by dermal fibroblasts in vivo and enhance in a paracrine fashion the proliferation of primary keratinocytes in vitro.

Pleiotrophin is a secreted, heparin-binding cytokine that was cloned as a proto-oncogene with the ability to transform NIH3T3 cells and to induce tumours in nude mice (Chauhan et al., 1993). PTN was reported to exert various biological functions: It acts as a mitogen for different fibroblastic and epithelial cell lines, and stimulates neurite outgrowth and angiogenesis (Deuel et al., 2002; Muramatsu, 2002). Because of its expression pattern during embryonic development, PTN was suggested to be involved in mesenchymal-epithelial interactions (Mitsiadis et al., 1995). Furthermore, a paracrine role for PTN as a mesenchymal growth factor for hepatocytes during liver regeneration has been described (Asahina et al., 2002).

SDF-1 (CXCL12) belongs to the intercrine family of cytokines and acts as a potent chemoattractant for hematopoietic and germ cells. It signals through binding to its exclusive receptor CXCR4 (LESTR; fusin) (Bleul et al., 1996; Oberlin et al., 1996), which functions as an HIV-1 co-receptor on lymphocytes (Lee et al., 1998) and has recently been shown to be highly upregulated on metastasizing breast cancer cells (Muller et al., 2001).

Although the role of SDF-1 and PTN in other systems has been studied extensively, to our knowledge this study presents, for the first time, evidence for the functional implication of these cytokines in the JUN-mediated dermal-epidermal crosstalk in skin.

Comparing the gene expression profiles of wild-type and *Jun*-deficient fibroblasts, we found PTN and SDF-1 to be strictly JUN dependent, as their mRNA levels were strongly reduced in cells lacking this transcription factor (Fig. 1) and reintroduction of a JUN expression vector restored *Ptn* and *Sdf-1* expression in mutant cells (see supplementary material Fig. S2). The impact of JUN on *Ptn* might be exerted directly through binding to the CRE (cAMP response element) site, which has been mapped within the promoter of the *Ptn* gene (Katoh et al., 1992). In contrast, the *Sdf-1* promoter is characterized by CpG-rich regulatory elements typical of house-keeping genes and does not contain obvious JUN binding sites (Shirozu et al., 1995). Thus, JUN appears to exert its function indirectly, presumably by controlling upstream components or co-factors necessary for SDF-1 expression.

In addition to PTN and SDF-1 being expressed in immortalized fibroblast cell lines, we showed that both cytokines are also expressed by dermal fibroblasts in vivo (Fig. 2; see also supplementary material Fig. S1). This is in agreement with previous studies, reporting on the expression of PTN in mouse embryos at sites of mesenchymal-epithelial

Journal of Cell Science

interactions (Mitsiadis et al., 1995) and in post-burn hypertrophic scar tissue in humans (Paddock et al., 2003). Keratinocytes might bind PTN via N-syndecan (Syndecan-3), which, in addition to the brain-specific anaplastic lymphoma kinase (ALK) and the protein tyrosine phosphatase ζ (PTP ζ), has been identified as functional PTN receptor (Kinnunen et al., 1998; Maeda and Noda, 1998; Stoica et al., 2001). For SDF-1, expression in skin has been described in stromal and endothelial cells (Fedyk et al., 2001), whereas its receptor CXCR4 was expressed on mesenchymal as well as epithelial cells (Pablos et al., 1999).

Here, we could demonstrate for the first time, that both cytokines, PTN and SDF-1, stimulate the proliferation of primary keratinocytes in vitro (Fig. 3). The mitogenic effect was most prominent in co-cultures containing *Jun*-deficient feeder cells that support keratinocyte growth with only low efficiency. When the strongly reduced PTN and SDF-1 production in *Jun*$^{-/-}$ fibroblasts was compensated by addition of the recombinant factors, proliferation of the keratinocytes was efficiently restored. Vice versa, application of neutralizing antibodies against SDF-1 or human CXCR4 specifically blocked keratinocyte growth in wild-type co-cultures. Importantly, both blocking antibodies do not interfere with the residual JUN-independent proliferation-promoting activity of *Jun*-deficient fibroblasts (Fig. 4) demonstrating that both proteins are specific components of the JUN-dependent mitogenic program. In contrast to SDF-1 and PTN a number of other factors, such as EGF, TGFα and bFGF, which were shown to promote keratinocyte proliferation (Schultz et al., 1987; O'Keefe et al., 1988; Marchese et al., 1990), were not, or only weakly able to rescue the proliferation defect of keratinocytes in *Jun*$^{-/-}$ fibroblast co-cultures (see supplementary material Fig. S3), suggesting that only a subset of mitogenic cytokines is able to exhibit proliferation-promoting activity in this system. Although an indirect effect of PTN and SDF-1 on keratinocytes, via induction of growth factors in fibroblasts, cannot be excluded, we favour the concept of direct action, since total proliferation-promoting activity is almost completely abolished in *Jun*-deficient fibroblasts, even in the presence of IL-1 and other stimuli produced by co-cultured keratinocytes (Szabowski et al., 2000).

It is important to note, however, that SDF-1 and PTN are not solely responsible for JUN-dependent mitogenic activity but appear to belong to a functional group of paracrine stimulators of keratinocyte proliferation, as do KGF and GM-CSF, which exert both redundant and non-overlapping functions. Therefore, it might not be surprising that the functional ablation of PTN (Zhang et al., 1999), KGF (Guo et al., 1993) or GM-CSF (Stanley et al., 1994; Dranoff et al., 1994) in mice does not result in an aberrant skin phenotype; SDF-1-deficient animals die as embryos from unrelated defects of the haematopoietic and nervous systems (Ma et al., 1998). Interestingly, expression of all four cytokines in fibroblasts is positively controlled by the proto-oncogene JUN. The apparent redundancy in diffusible factors regulating epithelial proliferation renders the system fail-safe and underscores the importance of skin integrity. This might be particularly important during the process of cutaneous wound healing, when rapid keratinocyte proliferation is required in order to cover the injured area. Indeed, we found that *Ptn* was induced during wound healing, as were *Kgf*, *Gro-1* and *Gm-csf* (Fig. 5A,B and data not shown) (Werner et al., 1992; Nanney et al., 1995; Mann et al., 2001). Interestingly, in contrast to the three latter cytokines which are all upregulated in response to IL-1 in fibroblasts (Fig. 1 and data not shown), neither *Ptn* nor *Sdf-1* are induced by this stimulus. Despite the important role of keratinocyte-derived IL-1 in initiating the cutaneous damage response, this finding is well in agreement with our recent observations, that IL-1 responsiveness and regulation in wounds are not necessarily correlated (Florin et al., 2004). Notably, *Ptn* expression by fibroblasts was increased in the presence of co-cultured keratinocytes (Fig. 5C), indicating that at least one other factor released from keratinocytes impacts on *Ptn* expression. Similarly, the downregulation of SDF-1 in co-cultures that is not observed in IL-1-treated cells supports the idea of a transregulatory control of cytokine expression in fibroblasts by factors other than IL-1. It is tempting to speculate that this inhibitory influence counteracts wound induction of SDF-1, resulting in its unchanged expression during cutaneous repair (Fig. 5). Interestingly, others have even observed a reduction of SDF-1 during wound healing and after treatment of fibroblasts with IL-1 and TPA (Jiang et al., 1994; Fedyk et al., 2001). Possibly, depending on the type of fibroblasts and the mouse strain used in the studies, composition of cell types in the wound and the experimental conditions, the molar ratio of positively and negatively acting signals on the SDF-1 promoter might differ. Probable factors exerting negative regulation on SDF-1 expression might include monocyte-derived TNF-α (Fedyk et al., 2001) or TGF-β, which is released from platelets in wounds and has been reported to repress SDF-1 expression in bone marrow stromal cells (Wright et al., 2003). Indeed, we observed a significant reduction of SDF-1 mRNA levels in TGF-β-treated fibroblasts (L.F. and P.A., unpublished). In contrast, JUN-independent expression of the SDF-1 receptor *Cxcr4* is weakly induced both by IL-1 treatment (Fig. 1B) (Gupta et al., 1998) and co-culture with keratinocytes (Fig. 5), suggesting that its upregulation in co-cultures might be attributable to IL-1 release by keratinocytes (Eddleston et al., 2002).

In summary, we have identified the cytokines PTN and SDF-1 as novel JUN target genes in fibroblasts that are expressed in the dermal compartment of the skin in vivo. Furthermore, we could demonstrate in feeder-layer co-cultures, that both factors enhance the growth of primary keratinocytes. Thus, PTN and SDF-1 represent two novel mesenchymal factors in the cytokine network, mediating the JUN-dependent impact of the fibroblasts on keratinocyte proliferation. Additionally, our observations not only substantiate the intriguing redundancy of factors by which epithelial integrity is controlled, but also hint towards a similar complex interplay at the level of keratinocytes, influencing in turn gene expression in fibroblasts.

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Journal of Cell Science

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