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CORRECTION

Loss of protein kinase Cε results in impaired cutaneous wound closure and myofibroblast function

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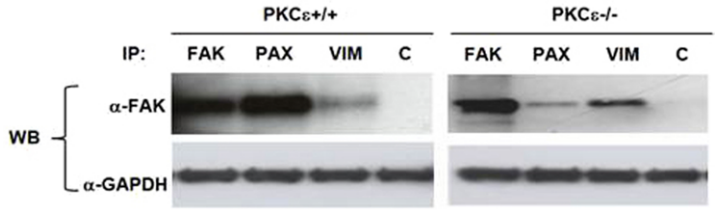
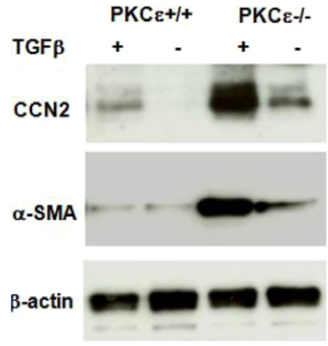
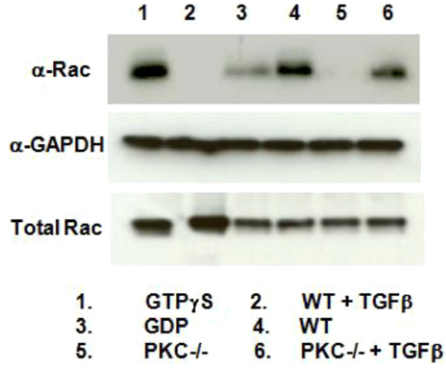
In Fig. 3B, a western blot showing the total input protein for the immunoprecipitation was not shown. The corrected figure with total protein levels identified is as shown below. An equal amount of total cell-derived protein were used to assess the interaction of FAK with paxillin (PAX) and vimentin (VIM).



In Fig. 4C, the western blotting images for the panels showing CCN2 and α-SMA were of poor quality. The corrected figure with high-quality images is shown below.



In Fig. 7B, the immunoprecipitation experiment for activated Rac was presented without control western blots showing total input protein and total level of Rac protein in the samples. The corrected figure is as shown below.



The authors apologise to the readers for any confusion that these errors might have caused.

Loss of protein kinase Cε results in impaired cutaneous wound closure and myofibroblast function

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Summary

Cutaneous wound repair requires the de novo induction of a specialized form of fibroblast, the α-smooth muscle actin (α-SMA)-expressing myofibroblast, which migrates into the wound where it adheres to and contracts extracellular matrix (ECM), resulting in wound closure. Persistence of the myofibroblast results in scarring and fibrotic disease. In this report, we show that, compared with wild-type littermates, PKCε$^{-/-}$ mice display delayed impaired cutaneous wound closure and a reduction in myofibroblasts. Moreover, both in the presence and absence of TGFβ, dermal fibroblasts from PKCε$^{-/-}$ mice cultured on fibronectin show impaired abilities to form 'supermature' focal adhesions and α-SMA stress fibers, and reduced pro-fibrotic gene expression. Smad3 phosphorylation in response to TGFβ1 was impaired in PKCε$^{-/-}$ fibroblasts. PKCε$^{-/-}$ fibroblasts show reduced FAK and Rac activation, and adhesive, contractile and migratory abilities. Overexpressing constitutively active Rac1 rescues the defective FAK phosphorylation, cell migration, adhesion and stress fiber formation of these PKCε$^{-/-}$ fibroblasts, indicating that Rac1 operates downstream of PKCε, yet upstream of FAK. These results suggest that loss of PKCε severely impairs myofibroblast formation and function, and that targeting PKCε may be beneficial in selectively modulating wound healing and fibrotic responses in vivo.

Key words: Myofibroblasts, TGFβ, Wound healing

Introduction

Tissue repair requires a well-defined series of events, starting with the plugging of the wound by a fibrin clot, and ending with the reconstitution of the epithelial barrier and the underlying connective tissue (Martin, 1997). During normal wound healing in adult animals, fibroblasts initially migrate into the wound area and synthesize a collagen- and cellular fibronectin-rich ECM (Eckes et al., 1999). This migration involves the extension of lamellopodia and filopodia, accompanied by the assembly of focal adhesions at the leading edge of the cell, and disassembly of the focal adhesions at the base of the protrusion. Regulation of focal adhesion formation and turnover is intimately related to the ability of the focal adhesions, through transmembrane integrins, to mediate contact with the extracellular matrix and the actin cytoskeleton (Burridge and Chrzanowska-Wodnicka, 1996). These focal adhesions typically contain β- and γ-cytoplasmic actins, α-SMA, α_v integrin, vinculin, paxillin, α-actinin, talin, focal adhesion kinase (FAK) and tyrosine-phosphorylated proteins (Dugina et al., 2001; Zamir and Geiger, 2001). These specialized fibroblasts are termed myofibroblasts, and generate the tensile forces required for wound closure. Abnormal persistence of the myofibroblast results in scarring and fibrotic disease (Gabbiani, 2003; Chen et al., 2005). Thus, selective modulation of myofibroblast action is expected to have profound impact on controlling tissue repair and fibrosis.

Focal adhesion proteins serve as a point of convergence for signals that result from stimulation of growth factor receptors (Turner, 2000). For example, paxillin provides a platform for protein tyrosine kinases such as FAK and SRC, which are activated as a result of adhesion or growth factor stimulation (Hagel et al., 2002). Fibroblasts lacking FAK or paxillin spread poorly and cannot migrate (Ilic et al., 1995; Webb et al., 2004). FAK-deficient fibroblasts actually possess more focal adhesions than do control cells, suggesting that FAK recruitment may be involved with the turnover of existing focal adhesions (Ilic et al., 1995). Indeed, FAK, paxillin, Src and ERK are required for focal adhesion disassembly at the base of extruding lamellopodia (Webb et al., 2004). FAK recruitment to paxillin is also required for Rac activation (Ishibe et al., 2004), which is essential for cell migration (Klemke et al., 1998). FAK activation is also required for matrix contraction (Midwood and Schwarzbauer, 2002).

Many details still remain to be discovered about the regulation and spatiotemporal relationship of the activation of focal adhesion components. For example, formation of focal adhesions is associated with the stimulation of protein kinase C (PKC) (Woods and Couchman, 1992), yet the contribution of PKC to focal adhesion formation is not yet understood.

PKC is a family of serine/threonine kinases that regulate a variety of cell functions including proliferation, gene expression, cell cycle, differentiation, cytoskeletal organization cell migration and apoptosis (Carter and Kane, 2004). The existence of multiple isozymes of PKC has raised the issue of whether each PKC isozyme has a specific function. Indeed, differences in PKC isozyme protein structure and substrate preferences have allowed the family to be divided into three groups (Way et al., 2000). The conventional PKC

isozymes (α, βI, βII, and γ) are Ca^{2+}-dependent and are activated by phospholipids and diacylglycerol. The novel isozymes (δ, ε, μ, and θ) are calcium insensitive, but phospholipid and diacylglycerol dependent. Finally, the atypical PKC isozymes (η and λ/ζ) are insensitive to both Ca^{2+} and diacylglycerol. Although the activation mechanism of the PKC isozyme family is clearly different among the three subgroups: conventional, novel and atypical PKC, whether or not each isozyme in a subgroup has a specific function or activation mechanism has not been clarified. Indeed, many PKCs display overlapping substrate specificities in vitro (Teicher, 2006). Overall, studies in which active or dominant-negative PKC isoforms are overexpressed in vitro have been imperfect in predicting the physiological consequences of loss of individual PKC isoforms in vivo (Teicher, 2006). There may be functional compensation by other PKC isoforms in vivo (Teicher, 2006). Moreover, data from dominant-negative or constitutively active studies can be misleading, as it is not known whether the overexpressed protein only impacts the isoform of interest or also affects other closely related PKC isoforms (Moscat et al., 2006). Furthermore, as PKC isozymes do not function in isolation but exist in complexes with other signaling molecules, overexpressed proteins may also impact the function of these additional molecules and hence indirectly affect cell function (Teicher, 2006). Thus, to identify the in vivo functions of individual PKC isoforms, the use of genetic models, such as knockout mice, is essential.

Protein kinase Cε (PKCε) contains an actin-binding motif that is unique to this individual member of the PKC gene family (Prekeris et al., 1998). In addition, thrombin-induced matrix contraction by fibroblasts is associated with the interaction of PKCε with α-SMA (Bogatkevich et al., 2003). Moreover, PKCε is required for the recycling of integrin β1, to which PKCε is bound via RACK (Besson et al., 2002), from inside the cell to the cell surface (Ivaska et al., 2002). Finally, PKCε is required for the ability of TNFα to cause phosphorylation of STAT1, a process mediated by integrins (Ivaska et al., 2003). These results suggest that PKCε may play a crucial role in stress fiber formation at focal adhesions. However, this hypothesis has yet to be tested. Indeed, the precise contribution of PKCε to focal adhesion formation in fibroblasts and in wound closure is not known. PKCε knockout mice are viable and appear essentially normal, but show elevated susceptibility to bacterial infection (Castrillo et al., 2001) and subtle cardiovascular defects in response to hypoxia (Saurin et al., 2001; Littler et al., 2003). In this report, we have used PKCε knockout mice to assess the contribution of protein kinase Cε to focal adhesion formation, adhesion, migration and ECM contraction and wound closure.

Results

Protein kinase Cε knockout mice show reduced wound closure and presence of α-SMA expressing myofibroblasts

To investigate whether $PKC\varepsilon^{-/-}$ mice displayed a delay in cutaneous wound closure, we subjected 8-week-old mice homozygous for deletions in the PKCε gene, or their wild-type littermates, to the dermal punch wound model of wound healing. We monitored wound closure over a 14-day period, and found that $PKC\varepsilon^{-/-}$ animals displayed a marked reduction in the rate of wound closure (Fig. 1A). We examined sections taken from isolated tissue from animals 14 days-post wounding and found that $PKC\varepsilon^{-/-}$ animals showed reduced presence of α-SMA-expressing myofibroblasts therein (Fig. 1B). Decreased α-SMA expression was verified using Western blot analysis if tissue extracts of wounds from $PKC\varepsilon^{+/+}$ and $PKC\varepsilon^{-/-}$ animals (Fig. 1C). These results indicate that the wound healing

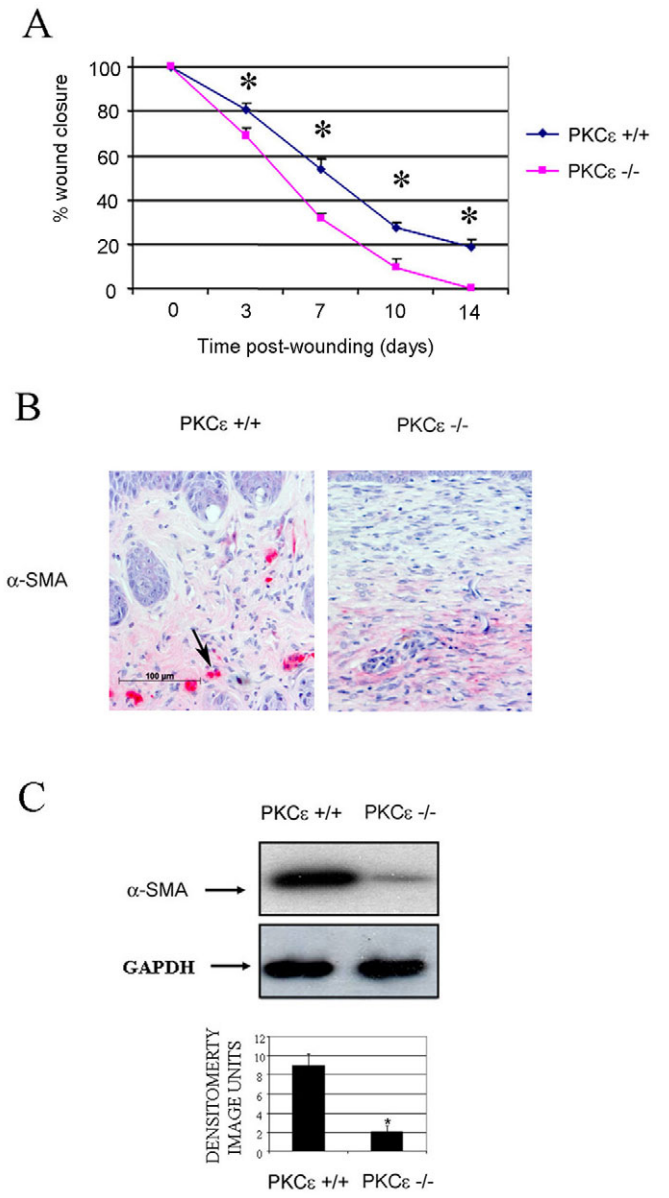

Fig. 1. $PKC\varepsilon^{-/-}$ mice show significantly reduced wound closure and presence of α-SMA-expressing myofibroblasts. (A) A 4 mm dermal punch was placed in the back of $PKC\varepsilon^{+/+}$ and $PKC\varepsilon^{-/-}$, and wound closure was measured. A total of six animals were analyzed (four wounds/animal). Eight-week-old females were assessed (*P<0.05, closure in $PKC\varepsilon^{-/-}$ significantly different from $PKC\varepsilon^{+/+}$ mice). (B) Anti-α-SMA (α-SMA) staining of 14 day wounds. Sectioning and staining revealed the reduced presence of α-SMA-positive myofibroblasts (arrow). Sections probed with the α-SMA antibody were counterstained with Hematoxylin. Scale bar: 100 μm. (C) Western blot analysis of lysates prepared from of $PKC\varepsilon^{+/+}$ and $PKC\varepsilon^{+/+}$ wounds (25 μg/lane). Densitometry represents mean±s.d. of eight independent experiments (i.e. extracts from eight separate wounds; *P<0.05).

phenotype of $PKC\varepsilon^{-/-}$ animals may arise due to impaired myofibroblast activity and function.

PKCε is required for 'supermature' focal adhesion formation

To begin to investigate the molecular defects underlying the inability of $PKC\varepsilon^{-/-}$ to rapidly close dermal wounds, fibroblasts were cultured from the dermis of mice homozygous for deletions

in the PKCε gene and wild-type littermates. Genotypes of the fibroblasts were confirmed by western blot analysis and RT-PCR (not shown). Our in vivo data indicated that loss of PKCε led to reduced tissue remodeling, wound closure and myofibroblast activity. To investigate this issue, we investigated whether loss of PKCε affected the ability of fibronectin to induce the appearance of α-SMA stress fibers and intensely vinculin-positive 'supermature' focal adhesions, key features of myofibroblasts (Hinz and Gabbiani, 2003). Fibronectin was used in our assays as this protein is a key component of the provisional matrix laid down in the initial phases of wound healing (Grinnell, 1984) and therefore provided an appropriate parallel approach to our in vivo wound healing studies. Intriguingly, we found that PKCε$^{-/-}$ fibroblasts cultured on fibronectin were able to recruit actin to form stress, as visualized by staining cells with phalloidin (Fig. 2). However, we found that α-SMA stress fiber networks, as visualized by staining of cells with an anti-α-SMA antibody, did not form in PKCε $^{-/-}$ fibroblasts (Fig. 2). To investigate whether PKCε was required for optimal focal adhesion formation by fibroblasts, we subjected PKCε$^{+/+}$ and PKCε$^{-/-}$ fibroblasts growing on fibronectin to indirect immunofluorescence with anti-focal adhesion kinase (FAK) and anti-vinculin antibodies. We found that vinculin and phospho-FAK staining were markedly reduced in intensity and number in PKCε$^{-/-}$ fibroblasts (Fig. 2). That intense vinculin staining did not appear in PKCε $^{-/-}$ fibroblasts is consistent with a failure of PKCε$^{-/-}$ cells to support formation of α-SMA stress fibers, as highly vinculin-positive focal adhesions are correlated with the larger, so-called 'supermature' α-SMA stress fiber-nucleating FAs (Dugina et al., 2001).

To confirm the notion that PKCε was required for focal adhesion and α-SMA stress fiber formation, cytoskeletal protein preparations were prepared from wild-type and PKCε knockout fibroblasts grown on fibronectin. The resultant protein preparations were subjected to western blot analysis with anti-α-SMA, anti-actin, anti-paxillin, anti-FAK, anti-Rac and anti-vinculin antibodies. Although these components of stress fiber and focal adhesion complexes were readily detected in the insoluble fraction of normal fibroblasts, suggesting that α-SMA stress fibers and focal adhesions were able to be formed in wild-type fibroblasts, α-SMA, paxillin, moesin and vinculin were found at reduced levels the insoluble fraction of PKCε knockout fibroblasts, giving further support to the notion that loss of PKCε impaired the formation of α-SMA stress fibers and focal adhesions (Fig. 3A). Consistent with the notion that an actin network formed in PKCε$^{-/-}$ fibroblasts, actin was recruited to the insoluble fraction of both PKCε$^{+/+}$ and PKCε$^{-/-}$ fibroblasts (Fig. 3A). Interestingly, however, FAK was found in the insoluble protein fraction, although it displayed reduced phosphorylation (Fig. 3A). These results suggest that the defect in FAK activation lies not at the level of FAK recruitment, but rather at the level of FAK activation. Consistent with this notion, immunoprecipitation experiments with an anti-paxillin antibody, which binds activated FAK and is required for focal adhesion assembly (Turner, 2000), showed that PKCε$^{-/-}$ fibroblasts showed markedly reduced binding between paxillin and FAK (Fig. 3B). Intriguingly, Rac, a protein activated and localized to focal adhesions upon adhesion and necessary for migration (Ishibe et al., 2004; Klemke et al., 1998), showed markedly reduced recruitment to the insoluble protein fraction (Fig. 3A).

To extend our analyses into the contribution of PKCε to fibroblast biology, we assessed whether PKCε-deficient fibroblasts showed an impaired response to the potent pro-fibrotic, myofibroblast-inducing cytokine TGFβ (Leask and Abraham, 2004). First, we used immunofluorescence and western blot analyses to show that the ability of TGFβ to induce α-SMA stress fiber formation was impaired in the absence of PKCε (Fig. 4A). In addition, we used western blot analysis to show that the ability of TGFβ to induce phosphorylation of Smad3 was impaired in the absence of PKCε (Fig. 4B). As these data suggested TGFβ-induced gene expression responses might be impaired in the absence of PKCε, we investigated whether loss of PKCε affected the ability of fibroblasts to induce the pro-fibrotic proteins CCN2, type I collagen, β1 integrin, α4 integrin and paxillin. We found that induction in response to TGFβ of all of these were impaired in PKCε-deficient fibroblasts (Fig. 4C). Finally, basal integrin, α-SMA and CCN2 expression were impaired (Fig. 4C). These results collectively imply that loss of PKCε significantly affects TGFβ signaling and myofibroblast induction in response to this growth factor.

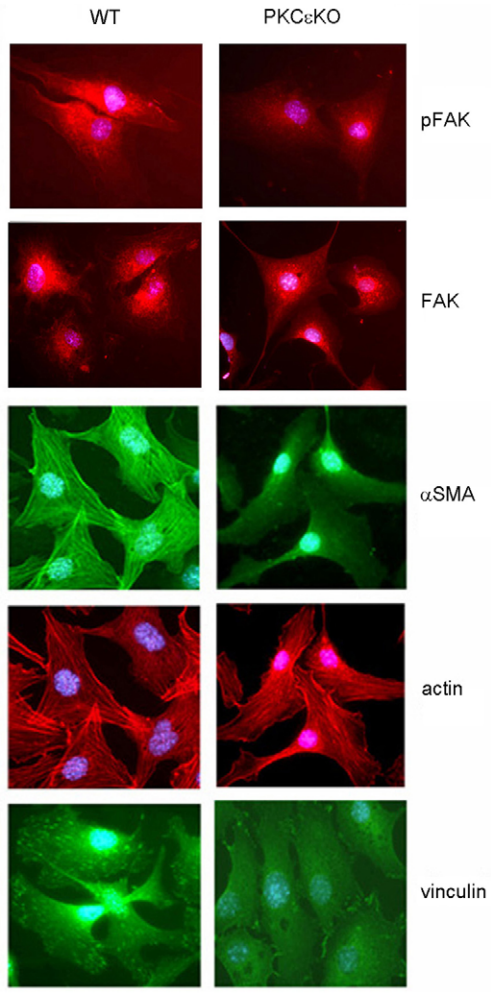

Fig. 2. PKCε$^{-/-}$ fibroblasts show reduced appearance of α-SMA stress fibers and 'supermature' focal adhesions on fibronectin. PKCε$^{+/+}$ (WT) and PKCε$^{-/-}$ fibroblasts cultured on fibronectin were fixed with paraformaldeyde and stained to detect phosphorylated FAK (pFAK), FAK, α-SMA, total actin, and vinculin. Cells were counterstained with DAPI to detect nuclei (blue stain). PKCε$^{+/+}$, but not PKCε$^{-/-}$, cells show foci of phospho-FAK staining (pFAK) or intense vinculin staining, which are characteristic of 'supermature' focal adhesions.

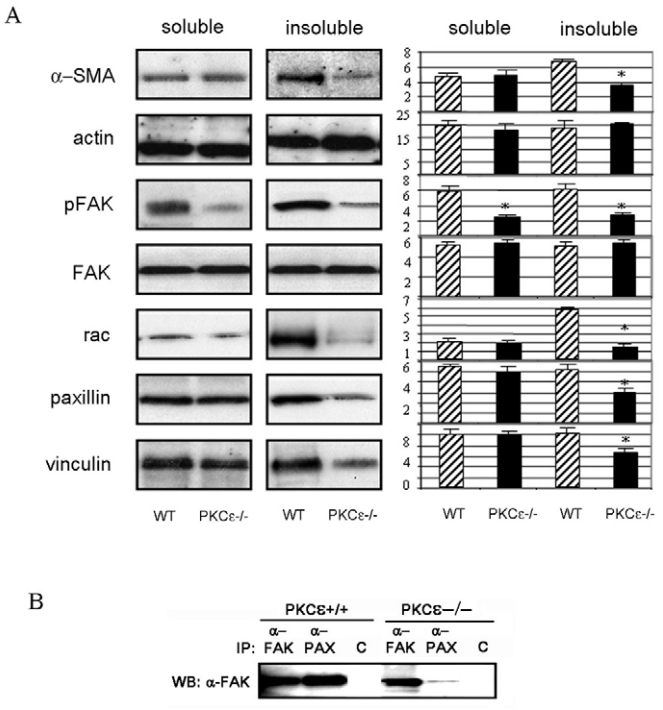

Fig. 3. PKCε$^{-/-}$ fibroblasts show reduced recruitment of focal adhesion components on fibronectin. (A) Compartmentalization of focal adhesion components in PKCε$^{+/+}$ (WT) and PKCε$^{-/-}$ fibroblasts. Cells were cultured until 80% confluence on fibronectin. Fibronectin is a major component of the provisional extracellular matrix deposited during wound healing (Grinnell, 1984). Triton-soluble and -insoluble protein extracts were prepared and subjected to SDS/PAGE as described in the Materials and Methods. Recruitment of FAK and β-actin is not affected. Western blot analyses (25 μg/lane) were then performed with anti-α-SMA, anti-β-actin, anti phospho-FAK, anti-FAK, anti-Rac1, anti-paxillin and anti-vinculin antibodies. There was reduced recruitment of Rac, paxillin, vinculin, phospho-FAK and α-SMA to the insoluble (cytoskeleton) fraction of the cell. Recruitment of FAK and β-actin was not affected. FAK phosphorylation was not completely impaired in the absence of PKCε. Densitometry was performed (n=3, average±s.d. is shown, *P<0.05 relative to wild-type control). (B) Interaction between paxillin and FAK in PKCε$^{+/+}$ and PKCε$^{-/-}$ fibroblasts. Cell extracts (25 μg) from cells cultured in an analogous fashion to those in A were immunoprecipitated with α-FAK or α-paxillin (α-PAX) or normal goat serum control (C), as indicated. The resultant protein extracts were subjected to SDS/PAGE, and immunoprecipitated FAK was detected with a α-FAK antibody by western analysis. FAK shows reduced interaction with paxillin in the absence of PKCε.

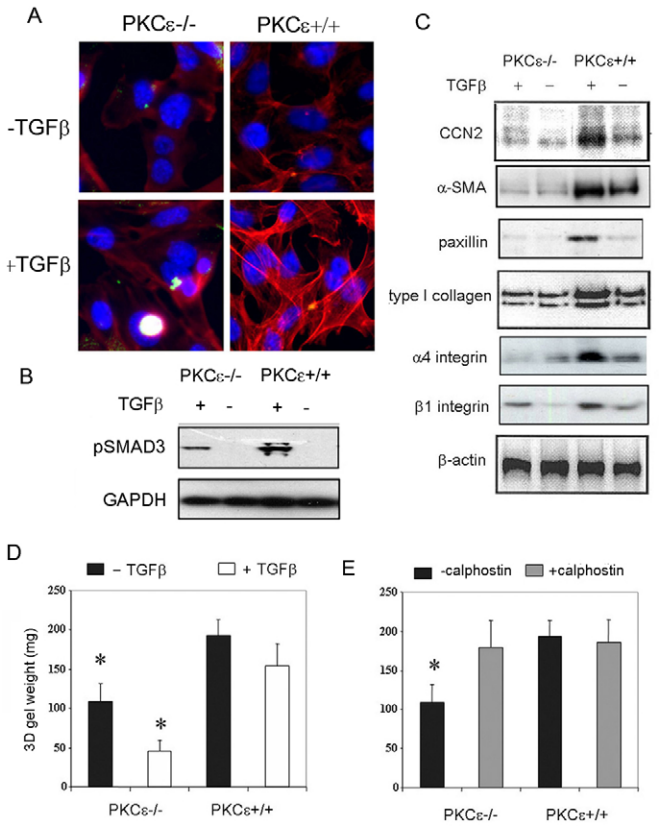

Fig. 4. Loss of PKCε reduces the ability of TGFβ to induce α-SMA stress fibers, Smad3 phosphorylation, pro-fibrotic protein expression and matrix contraction in fibroblasts. (A) Immunofluorescence analysis. PKCε$^{+/+}$ and PKCε$^{-/-}$ fibroblasts were cultured on fibronectin, serum starved for 18 hours and treated for an additional 24 hours with or without added TGFβ1 (4 ng/ml). Cells were fixed with paraformaldeyde and stained to detect α-SMA. Cells were counterstained with DAPI to detect nuclei. (B) Western blot analysis and Smad phosphorylation. PKCε$^{+/+}$ and PKCε$^{-/-}$ fibroblasts were cultured on fibronectin, serum starved for 18 hours and treated for an additional 0.5 hours with or without added TGFβ1 (4 ng/ml). Proteins were extracted, using 2% SDS, from cells. Equal amounts of protein (20 μg) were subjected to SDS/PAGE and western blot analyses with the antibodies indicated. (C) Western blot analysis and TGFβ-induced gene expression. PKCε$^{+/+}$ and PKCε$^{-/-}$ fibroblasts were cultured on fibronectin, serum starved for 18 hours and treated for an additional 24 hours with or without added TGFβ1 (4 ng/ml). Proteins were extracted, using 2% SDS, from cells. Equal amounts of protein (20 μg) were subjected to SDS/PAGE and western blot analyses with the antibodies indicated. Note that basal protein expression is reduced in the absence of PKCε. Moreover, expression of proteins in response to TGFβ is also impaired. (D) Floating collagen gel contraction assay. PKCε$^{+/+}$ and PKCε$^{-/-}$ fibroblasts were seeded into a floating collagen gel matrix, and incubated for 24 hours in the presence and absence of added TGFβ1 as described in the Materials and Methods. The weight of the contracted collagen gel was then measured. Cells were assayed in triplicate; experiments were performed thrice. Values shown are average±s.d. *significantly different from control (P<0.05). (E) PKCε$^{+/+}$ and PKCε$^{-/-}$ fibroblasts were seeded into a floating collagen gel matrix, in the presence or absence of the protein kinase C inhibitor calphostin C and analyzed as in C.

Protein kinase Cε knockout fibroblasts show reduced contraction and adhesion of ECM

To provide a functional context for our observations, we compared the abilities of PKCε$^{+/+}$ and PKCε$^{-/-}$ fibroblasts to contract a collagen gel matrix, suspended in medium, over a 24 hour period (Grinnell, 2003; Shi-wen et al., 2004). We found that PKCε$^{-/-}$ fibroblasts exhibited reduced ability to contract a floating collagen gel matrix both in the presence and absence of added TGFβ1 (Fig. 4D). Consistent with this observation, the ability of normal fibroblasts to contract a collagen gel was reduced by a general protein kinase C inhibitor, calphostin (Fig. 4E). To extend these data, we conducted a contraction assay using a fibroblast-embedded collagen gel matrix, which is fixed at one end and attached to a force monitor (Eastwood et al., 1994). Loss of PKCε resulted in an impaired ability of fibroblasts to generate contractile force across a fixed collagen gel lattice containing fibroblasts (Fig. 5A). We then compared the abilities of PKCε$^{+/+}$ and PKCε$^{-/-}$ fibroblasts to adhere to fibronectin, a key component of the provisional matrix deposited in the initial stages of wounding (Grinnell, 1984), and type I collagen. We found that PKCε$^{-/-}$ fibroblasts, relative to PKCε$^{+/+}$ fibroblasts, displayed markedly reduced abilities to adhere to fibronectin and type I collagen (Fig. 5B). Collectively, these results

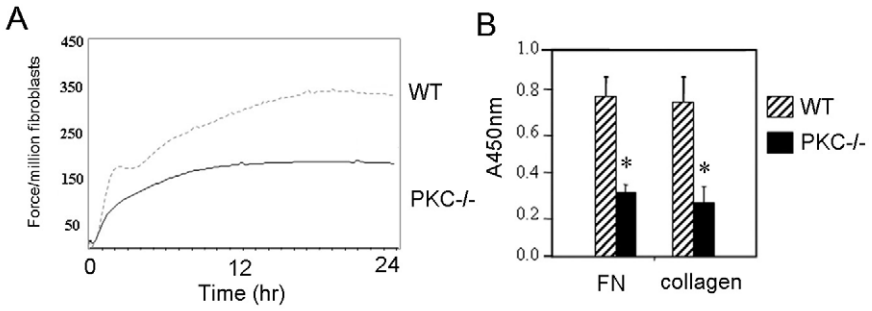

Fig. 5. Loss of PKCε results in a reduced ability of fibroblasts to contract and adhere to matrix. (A) Contraction of a collagen gel matrix: FPCL analysis. The effect of loss of PKCε expression on contractile force generated by fibroblasts in a fixed, tethered floating collagen gel lattice was investigated using a Culture Force Monitor (Eastwood et al., 1994). Force generated by PKCε$^{+/+}$ and PKCε$^{-/-}$ fibroblasts was assessed over 24 hours. Traces are the result of using 5 ml of collagen gel at a density of 1 million cells per ml of collagen gel in 2% serum. A representative trace is shown (n=3). The higher contractile forces generated by cells derived from PKCε$^{+/+}$ in comparison with PKCε$^{-/-}$ fibroblasts. (B) Loss of PKCε results in a reduced ability of fibroblasts to adhere to extracellular matrix. Adhesion of PKCε$^{+/+}$ and PKCε$^{-/-}$ fibroblasts on fibronectin (FN) and type I collagen (col) was assessed as described in the Materials and Methods. Cells were detached from plates with EDTA, and equal numbers of cells were placed into individual wells of 96-well plates. Adherent cells were detected (Absorbance 450) 45 minutes post-adhesion (average±s.d., n=3). *P<0.05 relative to wild-type control.

are consistent with the wound healing phenotype of the PKCε$^{-/-}$ mice, and suggest PKCε is required for crucial features of wound closure, namely the ability of fibroblasts to adhere to and contract ECM.

Protein kinase Cε knockout fibroblasts show reduced migratory ability that depends on Rac activation

To extend these studies, we compared the abilities of PKCε$^{+/+}$ and PKCε$^{-/-}$ fibroblasts to migrate on fibronectin by employing a so-called scratch wound assay in which a 'wound' is introduced, by a pipette, in a confluent cell monolayer (Kinsella and Wight, 1986). Both in the presence and absence of added TGFβ, we found that PKCε$^{-/-}$ fibroblasts showed greatly reduced migratory ability compared with PKCε$^{+/+}$ fibroblasts (Fig. 6). Collectively, these results are consistent with the notion that loss of PKCε results in impairment of the ability of fibroblasts to participate in wound healing responses. Our previous observation that FAK, but not Rac, was appropriately recruited to the cytoskeleton in PKCε$^{-/-}$ fibroblasts (Fig. 3) led us to investigate further

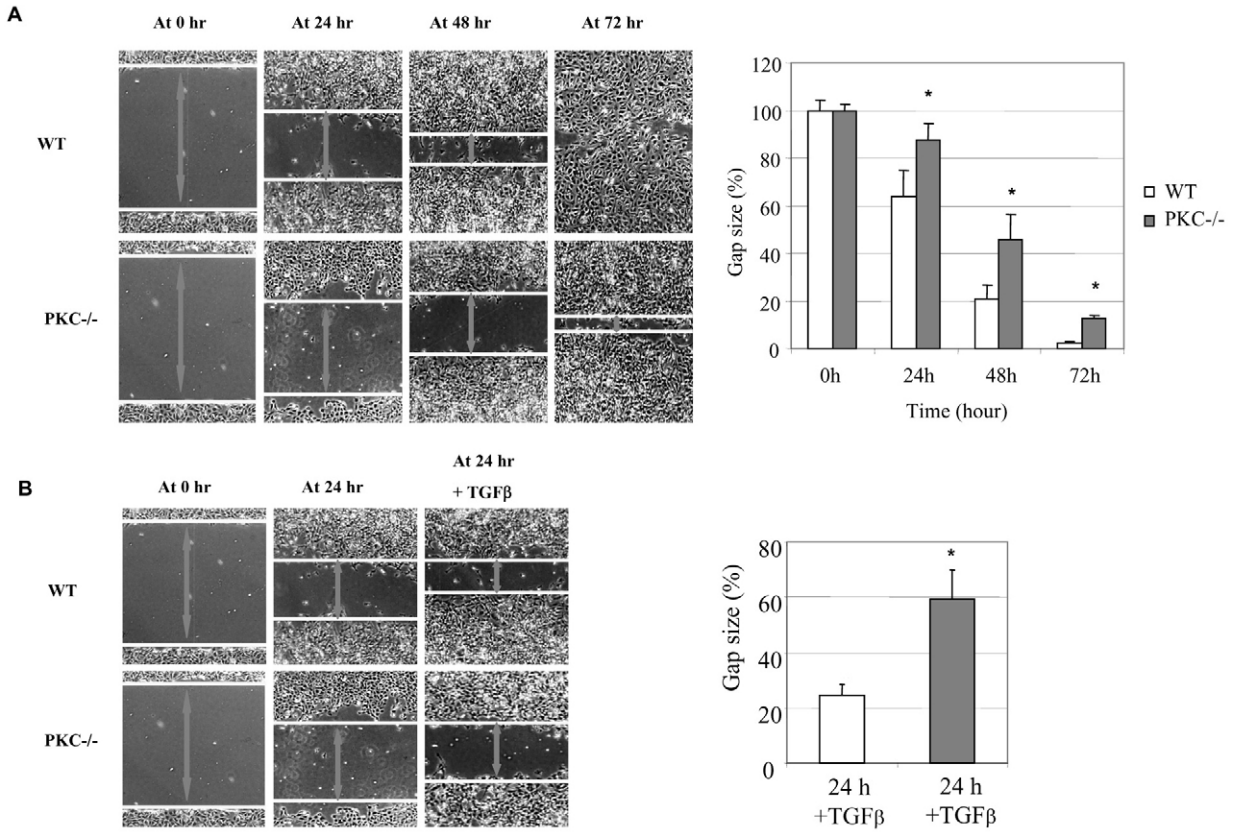

Fig. 6. Loss of PKCε results in a reduced ability of fibroblasts to migrate on extracellular matrix. (A) As described in the Materials and Methods, migration was measured using a scratch wound assay. PKCε$^{+/+}$ and PKCε$^{-/-}$ fibroblasts were cultured on fibronectin until confluence, and a linear scrape was made across the cell layer. Migration was monitored for the times indicated. Three independent experiments were performed Gap size expressed as a percentage of the original wound is shown (average±standard deviation) (*P<0.05 relative to wild-type control). (B) Migration was monitored as in A, in the presence and absence of added TGFβ1 (4 ng/ml). Quantitiative densitometry data are indicated on the right. Gap size expressed as a percentage of the original wound is shown (average±s.d.), *P<0.05 relative to wild-type control. Three independent experiments were performed.

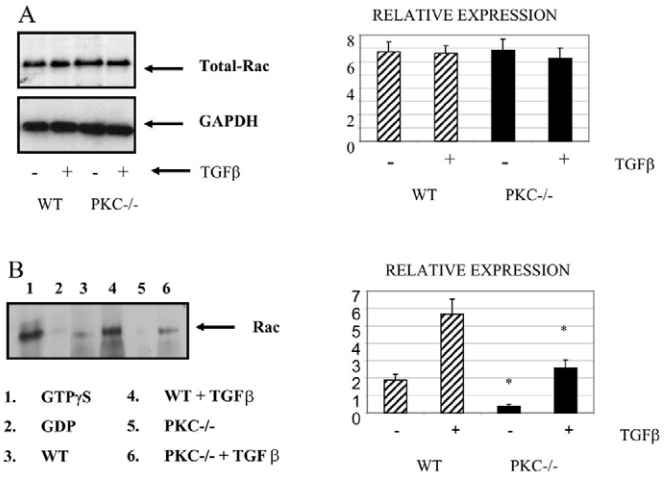

Fig. 7. Loss of PKCε results in impaired Rac signaling in fibroblasts. (A) Rac is expressed equally in PKCε$^{+/+}$ (WT) and PKCε$^{-/-}$ fibroblasts. Cells were loaded with or without GTPγS (positive control, to assess maximal Rac activation) and in the presence or absence of TGFβ (4 ng/ml, 1 hour). Cell lysates were subjected to SDS/PAGE and western blot analysis with an anti-Rac antibody. Experiments were performed six independent times. Quantitiative densitometry data are indicated on the right. (B) Rac activity assay. Input protein assessed in parallel with A was used in the standard Rac activity assay, as described in the Materials and Methods. Rac-GTP was immunoprecipitated from cell lysates. The precipitated Rac-GTP was detected by immunoblot analysis using anti-Rac. GTPγS (lane 1), GDP (lane 2), PKCε$^{+/+}$ (WT, lane 3), PKCε$^{+/+}$ (WT)+ TGFβ (lane 4); PKCε$^{-/-}$ (lane 5); PKCε$^{-/-}$ + TGFβ (lane 6). Experiments were performed three independent times. Quantitative densitometry data are indicated on the right (*P<0.05 relative to wild-type control).

the role of Rac in the phenotype of PKCε$^{-/-}$ fibroblasts. We then used a standard Rac activity assay to show that PKCε$^{-/-}$ fibroblasts, while displaying equal levels of Rac expression to wild-type cells with and without a 1-hour treatment with TGFβ, displayed markedly reduced Rac activity both in the presence and absence of added TGFβ (Fig. 7A,B).

Based on these results, we reasoned that Rac might not be effectively activated in PKCε$^{-/-}$ fibroblasts, and that the inability of FAK to be phosphorylated may be due to a markedly reduced level of Rac activity in PKCε$^{-/-}$ fibroblasts. We used western blot analysis to show that PKCε$^{-/-}$ fibroblasts cultured on fibronectin showed markedly endogenous reduced FAK phosphorylation (Fig. 8A). To assess whether the lower level of Rac contributed to the reduced FAK phosphorylation and to the reduced migration observed in PKCε$^{-/-}$ fibroblasts, we transfected an expression vector encoding constitutively activated Rac into PKCε$^{-/-}$ fibroblasts. Overexpression of constitutively activated Rac1 enhanced FAK phosphorylation and the reduced integrin β1 expression in PKCε$^{-/-}$ fibroblasts (Fig. 8A). Moreover, constitutively active Rac1 restored the ability of PKCε$^{-/-}$ fibroblasts to adhere to fibronectin and collagen, to possess abundant actin stress fibers (Fig. 8B,C) and to migrate (Fig. 8D). Thus, we concluded that PKCε was required for the activation of Rac, which was in turn required for maximal activation of FAK. It was still possible that the defect in PKCε$^{-/-}$ cells was due to the reduction of integrin expression (Fig. 4B) rather than to the inability of Rac to be activated. To investigate this possibility, we showed that Rac inhibition could reduce integrin β1 expression in PKCε$^{+/+}$ cells, indicating that Rac activation is located upstream of integrin β1 expression (Fig. 9A). Moreover, Rac inhibition had a similar effect on wild-type fibroblasts to the loss of PKCε, namely Rac inhibition resulted in reduced α-SMA expression (Fig. 9A), ECM contraction (Fig. 9B) and cell migration (Fig. 9C). Collectively, our results are consistent with the notion that the markedly reduced ability of focal adhesions to be properly formed in PKCε$^{-/-}$ fibroblasts (owing to an inability to properly activate Rac and, as a result, to phosphorylate FAK) contributes to defective wound healing observed in PKCε$^{-/-}$ mice.

Discussion

Although it has been shown that PKCε is activated upon cell adhesion, the function of PKCε in focal adhesion formation and function is unknown. In this report, we test the effect of loss of PKCε in fibroblasts and show that recruitment of focal adhesion components to the cytoskeleton and phosphorylation of FAK was impaired in PKCε knockout fibroblasts. Consistent with this idea, PKCε$^{-/-}$ mice showed reduced rates of wound closure, cellularization and ECM deposition. In addition, isolated PKCε$^{-/-}$ fibroblasts showed reduced focal adhesion formation and migration and contraction of ECM. PKCε$^{-/-}$ fibroblasts showed reduced adhesive signaling, as visualized by FAK and Rac activation. As transfection of Rac at least partially restored the defects in FAK phosphorylation and migration in the PKCε$^{-/-}$ fibroblasts, suggesting that activation of Rac is downstream of PKCε but upstream of FAK. These results are consistent with recent findings that Rac is required for deformation-induced FAK phosphorylation (Chaturvedi et al., 2007) and that loss of Rac1 in keratinocytes results in delayed wound closure (Tscharntke et al., 2007). However, perhaps our most crucial finding was that although overall formation of the normal actin cytoskeleton was not impaired in PKCε knockout fibroblasts, formation of the highly-contractile α-SMA cytoskeleton was markedly reduced in the absence of PKCε. Similar to this observation, PKCε$^{-/-}$ fibroblasts were able to form normal focal adhesions and actin fibers, but not the characteristic 'supermature' focal adhesions, as visualized by intense vinculin staining, of myofibroblasts (Dugina et al., 2001). It should be pointed out that the overall rate of wound closure was impaired in the absence of PKCε. Our results indicate that a crucial feature of this defect arises because of impaired myofibroblast formation. Although this defect is likely to have an indirect effect on the rate of re-epithelialization, we cannot exclude the possibility that loss of PKCε may also directly affect keratinocytes function. Evaluating this latter hypothesis is beyond the scope of our current study.

Fibrotic diseases are characterized by the failure to terminate normal tissue repair and the persistence of myofibroblasts within lesions (Gabbiani, 2003; Shi-wen et al., 2004; Chen et al., 2005). Myofibroblast formation can be driven by many processes, including tension, TGFβ, thrombin, ET1 and CCN2 (Arora et al., 1999; Hinz et al., 2001; Shephard et al., 2004; Shi-wen et al., 2004; Shi-wen et al., 2006a; Shi-wen et al., 2006b; Chen et al., 2005; Uehta et al., 1997; Leask and Abraham, 2004; Leask and Abraham, 2006; Goffin et al., 2006; Kennedy et al., 2007). These processes cooperate in inducing myofibroblast persistence within the milieu of tissue repair and fibrosis (Arora et al., 1999; Hinz et al., 2001; Shephard et al., 2004; Shi-wen et al., 2006a; Shi-wen et al., 2006b). Thus, our results showing that PKCε mediates myofibroblast formation downstream are a useful first step in identifying a target suitable for anti-fibrotic drug intervention. Moreover, several growth factors and cytokines that promote myofibroblast formation are pleiotropic, which is reflected in the phenotype of their respective knockout mice being lethal or displaying severe developmental defects (e.g. Ivkovic et al.,

2003; Hines et al., 1994; Kurihara et al., 1994). Conversely, PKCε$^{-/-}$ mice are viable and healthy, unless subjected to particular stresses, such as subcutaneous injection of bacteria or hypoxia, in which case they show increased susceptibility to infection and reduced vasoconstriction, respectively (Castrillo et al., 2001; Saurin et al., 2002; Littler et al., 2003). These data, combined with our observations that loss of PKCε results in impaired tissue repair and myofibroblast function, suggest that compared to the other pro-fibrotic targets described above, PKCε may be a suitable selective target for controlling tissue repair and in alleviating pathological scarring in vivo. Direct testing of this hypothesis using animal models of fibrosis is under way, but is beyond the scope of our current study. However, it is interesting to note that we have recently demonstrated that mice possessing a fibroblast-specific deletion of Rac1 are resistant to bleomycin-induced skin fibrosis (Liu et al., 2008).

In conclusion, our results showing that PKCε is required for normal tissue repair and myofibroblast formation provide new insights into the function of specific PKC isoforms and into the integrated molecular mechanisms that underlie normal tissue repair.

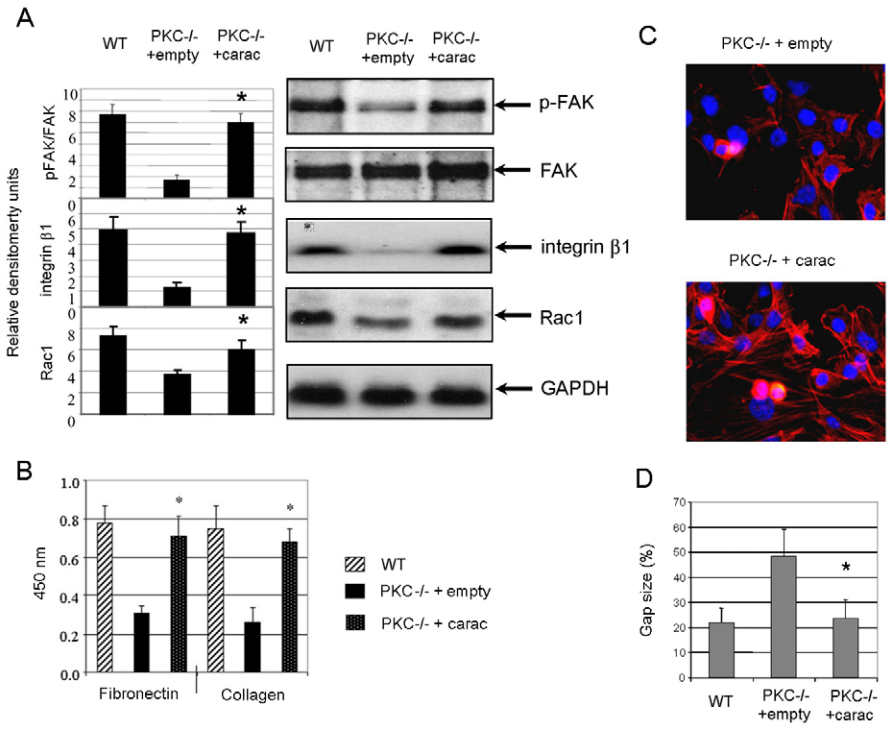

Fig. 8. Phenotype of PKCε$^{-/-}$ fibroblasts rescued by transfection with expression vector encoding constitutively active Rac1. (A) FAK phosphorylation and integrin β1 expression were analyzed as described in the Materials and Methods. Western blot analysis was used to detect phosphorylated FAK, total FAK, integrin β1 and total Rac1 in PKCε$^{+/+}$ and PKCε$^{-/-}$ fibroblasts cultured on fibronectin. Transfection of constitutively active Rac (caRac), compared with empty expression vector (empty), increases phosphorylation of FAK and integrin β1 expression in PKCε$^{-/-}$, as revealed by western blot analysis. Cell extracts were prepared 24 hours post-transfection. FAK phosphorylation and integrin β1 expression were not completely impaired in the absence of PKCε. Quantitation of three independent experiments was performed using densitometry. Average±s.d. is shown (n=3, *P<0.05). (B) Adhesion. Cells cultured on fibronectin were transfected with empty expression vector or expression vector encoding activated Rac1 (caRac). Adhesion of PKCε$^{+/+}$ (WT) and PKCε$^{-/-}$ fibroblasts on fibronectin and type I collagen was monitored. Cells were detached from plates by using EDTA, and equal numbers of cells were placed into individual wells of 96-well plate. Adherent cells were detected (Absorbance 450) 45 minutes post-adhesion (average±s.d., n=3). (C) Stress fiber formation. PKCε$^{+/+}$ and PKCε$^{-/-}$ fibroblasts cultured on fibronectin were fixed with paraformaldeyde and stained to detect α-SMA. Cells were counterstained with DAPI to detect nuclei (blue stain). PKCε$^{-/-}$ cells transfected with caRac1 show stress fibers. (D) Migration of PKCε$^{+/+}$ (WT) and PKCε$^{-/-}$ fibroblasts on fibronectin was monitored, using a scratch wound assay as described in the Materials and Methods. Photographs were taken 72 hours post-transfection, 48 hours after introduction of the scratch wound. Gap size expressed as a percentage of the original wound is shown (average±s.d.; n=3). Overexpression of Rac1 significantly increased migration of PKCε$^{-/-}$ fibroblasts (Student's paired t-test, *P<0.05) compared with the appropriate control.

Materials and Methods

Punch wound assay

All animal protocols were approved by the local animal ethics committee at the Royal Free and University College Medical School. Female PKCε$^{+/+}$ and PKCε$^{-/-}$ mice aged 8 weeks, a kind gift from Peter Parker (University College London) (Castrillo et al., 2001), were anesthetized with Avertin (500 mg/kg). The back was shaved and cleaned with alcohol. Four equidistant 4 mm^3 full-thickness excisional wounds were made on either side of the midline of six animals/data set. Mice were sacrificed by CO_2 euthanasia after 3, 7, 10 and 14 days. Immediately after sacrificing, the wound diameter was measured and wounds were photographed before samples were collected for histology, immunohistochemistry and protein extraction.

Cell culture, immunofluorescence and western analysis

Fibroblasts were isolated from explants (4- to 6-week-old animals) as previously described (Chen et al., 2004). To mimic conditions in the provisional matrix post-wounding (Grinnell, 1994), cells were cultured on fibronectin (4 mg/ml Sigma) (Chen et al., 2004). Cells were subjected to indirect immunofluorescence analysis as previously described (Chen et al., 2004) using anti-α-SMA, rhodamine phalloidin (Sigma), anti-vinculin or anti-phospho-FAK (Cell Signaling), followed by an appropriate secondary antibody (Jackson ImmunoResearch). Photomicrographs were taken (Zeiss Axiphot) using a digital camera and Adobe Photoshop. Where indicated, cells were stained with DAPI (Molecular Probes, Eugene, OR) to detect nuclei. Alternatively, cells were lysed in 2% SDS, proteins quantified (Pierce) and subjected to western blot analysis as previously described (Shi-wen et al., 2004; Shi-wen et al., 2006a) with anti-β1 integrin, anti-α4 integrin (Santa Cruz), anti-CCN2 (Abcam), anti-paxillin (Cell Signaling), anti-type I collagen (Biodesign), anti-phosphoo-Smad3 (Rockland), anti-α-SMA or β-actin (Sigma) antibodies. Where indicated, cells were pretreated with the Rac inhibitor NSC23766 (100 μM, 1 hour, Calbiochem). Where indicated, cell extraction was performed in Triton lysis buffer (to generate a Triton-soluble fraction) and the Triton insoluble (cytoskeletal) fraction was solubilized in 2% SDS prior to western blot analysis. For the Rac rescue experiment, an expression vector encoding constitutively active Rac (V12rac1) under the control of the cytomegalovirus promoter (courtesy of Alan Hall, University College London) or an empty expression vector was transfected into cells using Fugene (Roche) at a ratio of 2 μg DNA:3 μl Fugene.

Tissue sectioning

Specimens were fixed after 24 hours in 10% neutral buffered formalin after which they were embedded in paraffin. Paraffin-embedded sections were cut (3 μm) on a Leica RM 2135 microtome. For α-SMA staining, an anti-α-SMA antibody (Chemicon: 1:50 dilution, 1 hour) was used followed by detection using Vectastain (Vector Labs), as described by the manufacturer. Samples were counterstained with Hematoxylin.

Rac activity assay

A standard commercially available Rac-GTP pulldown assay was used (Upstate Biotechnology; Lake Placid, NY). Rac activity assays were performed as described by the manufacturer. Briefly, cells were grown in 6 cm dishes. Cells were lysed in a buffer containing NP-40. A PAK-GSH fusion protein bound to agarose beads was added, and active Rac, which binds PAK-GSH, was separated by repetitive centrifugation and washing. After the specimens were boiled in Laemmli buffer, they were subjected to SDS-PAGE and Rac was quantified by Western blot analysis. In some experiments, cell lysates were stimulated with GTPS (100 μmol/l) to obtain maximal activation of Rac.

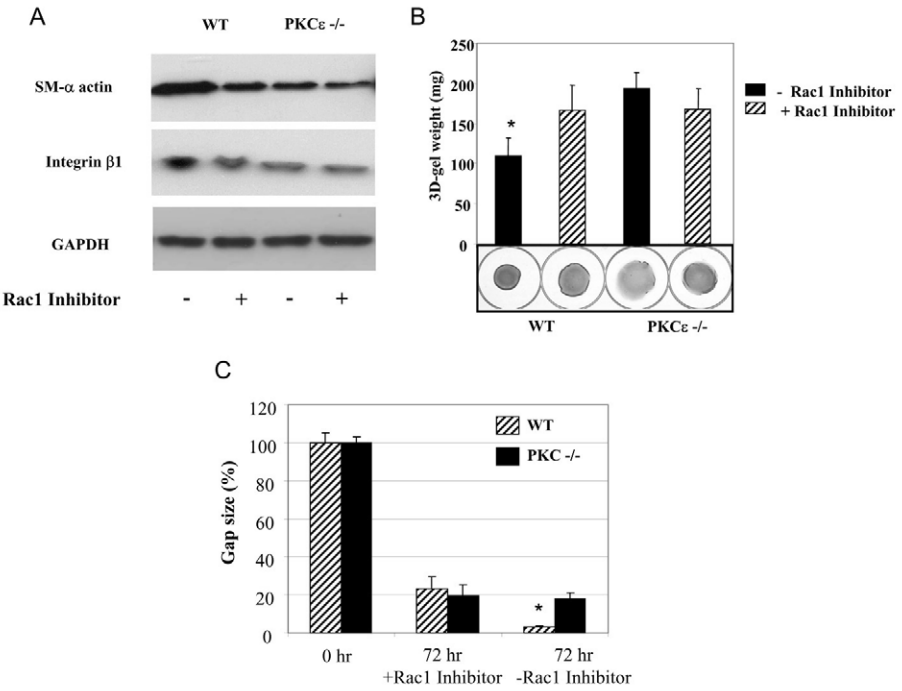

Fig. 9. Rac inhibition reduces integrin β1 expression in PKCε$^{+/+}$ fibroblasts. (A) As described in the Materials and Methods, western blot analysis was used to detect integrin β1 expression in PKCε$^{+/+}$ and PKCε$^{-/-}$ fibroblasts. Cells were incubated in the presence or absence of the Rac inhibitor NSC23766 (100 μM, 1 hour). Rac inhibition reduced integrin β1 expression in PKCε$^{+/+}$, but not in PKCε$^{-/-}$, cells, indicating Rac operates upstream of integrin β1 expression and, in this context, in a PKCε-dependent fashion. Rac inhibition also reduced α-SMA protein expression in PKCε$^{+/+}$, but not PKCε$^{-/-}$, cells. (B) As described in the Materials and Methods, a floating collagen gel contraction assay was used to detect ECM contraction by PKCε$^{+/+}$ and PKCε$^{-/-}$ fibroblasts over a 24-hour period. Cells were incubated in the presence or absence of the Rac inhibitor NSC23766 (100 μM). Contraction was observed only in untreated PKCε$^{+/+}$ cells (*P<0.05). (C) As described in the Materials and Methods, a cell migration assay was performed on PKCε$^{+/+}$ and PKCε$^{-/-}$ fibroblasts over a 72-hour period in the presence or absence of the Rac inhibitor NSC23766 (100 μM). Gap size expressed as a percentage of the original wound is shown (average±s.d.). The presence of the Rac inhibitor reduced migration of PKCε$^{+/+}$ cells (*P<0.05).

Adhesion assay

Fibroblasts were isolated and cultured as described above. Adhesion assays were performed essentially as previously described (Chen et al., 2004). Wells of 96-well plates overnight, 4°C, with 10 μg/ml fibronectin (Sigma) or type I collagen (First Link) in 0.5% bovine serum albumin (BSA), 1 PBS. Wells were blocked for 1 hour in 10% BSA in PBS at room temperature. Fibroblasts were harvested with 2 mM EDTA in PBS (20 minutes, room temperature), washed twice with DMEM serum-free medium containing 1% BSA (Sigma, St Louis, MO), resuspended in the same medium at 2.5×10^5 cells/ml and 100 μl of suspension was incubated in each well for 1 hour. Non-adherent cells were subsequently removed by washing with PBS. To detect cell adhesion, an acid phosphatase assay was used, adherent cells were quantified by incubation with 100 μl substrate solution (0.1 M sodium acetate, pH 5.5; 10 mM *–p*-nitophenylphosphate and 0.1% Triton X-100) for 2 hours at 37°C. The reaction was stopped by the addition of 15 μl 1N NaOH/well and A_{450} was measured. Comparison of adhesive abilities was performed by using Student's unpaired *t*-test. P<0.05 was considered to be statistically significant.

Collagen gel contraction

Experiments were performed essentially as described previously (Shi-wen et al., 2004). Briefly, 24-well tissue culture plates were pre-coated with BSA. Cells were used at passage 3. Trypsinized fibroblasts were suspended in MCDB medium (Sigma) and mixed with collagen solution [one part of 0.2 M N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), pH 8.0; four parts collagen (Vitrogen-100, 3 mg/ml) and five parts of MCDB X 2] yielding a final concentration of 80,000 cells per ml and 1.2 mg/ml collagen. Collagen/cell suspension (1 ml) was added to each well. After polymerization, gels were detached from wells by adding 1 ml of MCDB medium. Contraction of the gel was quantified by loss of gel weight and decrease in gel diameter over a 24-hour period

Fibroblast populated collagen lattices (FPCL)

Measurement of contractile force generated within a three-dimensional, tethered FPCL was performed as described previously (Eastwood et al., 1994; Shi-wen et al., 2004) Using 110^6 cells/ml of collagen gel (First Link, UK), we measured the force generated across the collagen lattice with a culture force monitor, which measures forces exerted by cells within a collagen lattice over 24 hours as fibroblasts attach, spread, migrate and differentiate into myofibroblasts. In brief, a rectangular fibroblast seeded collagen gel was cast and floated in medium in 2% FCS in the presence or absence of TGFβ1 (4 ng/ml), while tethered to two flotation bars on either side of the long edges, in turn attached to a ground point at one end and a force transducer at the other. Cell-generated tensional forces in the collagen gel are detected by the force transducer and logged into a personal computer. Graphical readings are produced every 15 seconds providing a continuous output of force (Dynes: 110^{-5}N) generated (Eastwood et al., 2004). The cells used in these experiments were passage matched; experiments were run in parallel and three independent times. A representative trace is shown.

Migration assays

For in vitro wounding (migration) experiments, cultured fibroblasts obtained from wild-type or PKCε KO mice were grown on fibronectin in 12-well plates. Medium was removed and cells were once rinsed with serum-free medium + 0.1% BSA and were cultured for 24 hours in serum-free medium + 0.1% BSA. The monolayer was artificially injured by scratching across the plate with a blue pipette tip (~1.3 mm width) (Kinsella and Wight, 1986). The wells were washed twice to remove detached cells or cell debris. The cells were then cultured in serum-free medium with or without added TGFβ1 (4 ng/ml R&D Systems). Mitomycin C (10 μg/ml) was always included in the media to prevent cell proliferation. After 48 hours, five representative images of the scratched areas under each condition were photographed.

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