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Disruption of Basal Jun-N-Terminal Kinase (JNK) Activity Differentially Affects Key Fibroblasts Functions Important for Wound Healing

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Abbreviations: ECM, extracellular matrix; JNK, c-Jun N-terminal kinase; TGF- β , transforming growth factor- β ; wt, wild-type

Running title: Basal JNK activity regulates fibroblast functions

Key Words Cell Motility, Cell Migration, Collagen Gene Expression, Jun N-Terminal Kinase, Wound Healing

SUMMARY

We used both a gene knockout approach and pharmacologic modulation to study the implication of the Jun-N terminal kinase (JNK) pathway in regulating fibroblast motility, capacity to contract mechanically unloaded collagen gels, and type I collagen gene expression *in vitro*. These parameters, important for tissue repair, are positively regulated by TGF- β , a cytokine viewed as playing a master role during wound healing. We demonstrate that basal JNK activity is critical for fibroblast motility, as (a) mouse embryo *jnk*^{-/-} fibroblasts exhibit significantly lower ability to close mechanically-induced cell layer wounds than their wild-type counterparts, and (b), wound closure by human dermal fibroblasts is dramatically impaired by the specific JNK inhibitor SP600125. *JunAA* fibroblasts, in which amino-acids Ser63 and Ser73 of c-Jun are replaced by two Ala residues so it cannot be phosphorylated by JNK, also exhibited impaired motility, suggesting that c-Jun phosphorylation by JNK is critical for fibroblast migration. In sharp contrast to their lesser motility on plastic, *jnk*^{-/-} and *junAA* fibroblasts contracted free-floating, mechanically unloaded, collagen lattices markedly faster than wild-type fibroblasts. Furthermore, basal mRNA steady-state levels for types I and III collagen genes were similar in *jnk*^{-/-} and wild-type fibroblasts. Likewise, over-expression of a dominant-negative mutant form of MKK4 in dermal fibroblasts did not affect collagen expression. We also demonstrate that basal JNK activity does not affect either TGF- β -induced collagen gene expression or lattice contraction, whereas on the other hand, the blockage of motility initiated by JNK inhibition cannot be overcome by TGF- β . Together these results demonstrate discrete, yet significant and highly specific, regulation of fibroblast functions important for wound healing by basal JNK activity.

INTRODUCTION

Efficient cutaneous wound repair implies carefully orchestrated molecular events allowing fibroblasts to migrate to the wound site(s), contract the wound, and synthesize extracellular matrix (ECM) to restore skin integrity. Cellular signals underlying such events are not fully understood but it is well accepted that transforming growth factor- β (TGF- β), by means of its pleiotropic activities, plays a central role in orchestrating the various phases of wound healing (1-3).

Extracellular stimuli elicit specific intracellular signals via activation of a family of so-called mitogen-activated protein kinases (MAP kinases), consisting of extracellular signal-regulated kinases (ERK), p38 MAP kinases, and the c-Jun NH₂-terminal kinases (4-6). These MAP kinases phosphorylate transcription factors within the cell nucleus, thereby activating a number of cellular functions, including proliferation, apoptosis, differentiation, and regulation of gene expression. It has been reported that the ERK and p38 MAP kinases are activated in fibroblasts during collagen matrix contraction under isometric tension (7), and that they cooperate in contraction-stimulated activation of the immediate early gene *c-fos*, although they are not required for lattice contraction *per se* (8). In mechanically unloaded collagen lattices, on the other hand, ERK signaling is disrupted, a mechanism that may be responsible for the entry of fibroblast into a quiescent state after several days under these conditions (9).

The JNK group of MAP kinases, also known as stress-activated kinases, are activated upon exposure of cells to cytokines, growth factors, and environmental stresses such as UV irradiation or heat shock (10). Three distinct genes, *jnk1*, *jnk2* and *jnk3*, have been identified to encode JNKs. The former two genes are ubiquitously expressed, whereas *jnk3* is selectively expressed in the heart, testis and brain. Dual Thr and Tyr phosphorylation of JNK by the MAP kinase kinases, MKK4 and MKK7, results in JNK activation and nuclear translocation.

In the nucleus JNKs phosphorylate transcription factors such as c-Jun (11), a process that leads to maximal transcriptional activity of the latter (12). Thus far, little is known about the role of JNK in the context of fibroblast ability to remodel collagen matrices, except that it was shown recently that JNK regulates the phenotypic modulation of lung fibroblasts into myofibroblasts induced by TGF- β (13), IL-4 and IL-13 (14).

Recent data from our laboratory have indicated that basal JNK activity in fibroblasts maintains a limited, yet significant, pool of phosphorylated c-Jun protein (15). In this report, using pharmacologic and genetic approaches aimed at interfering with basal JNK activity, we demonstrate a critical role for the latter in allowing fibroblast motility, whereas it inhibits their ability to contract free-floating collagen matrices, and does not modify the expression of fibrillar collagen genes, or their modulation by TGF- β . Details are provided herein.

MATERIALS AND METHODS

Cell Cultures-Human dermal fibroblasts were established by explanting neonatal foreskins. Immortalized fibroblast cell lines derived from wild-type (wt) and $jnk1^{-/-}jnk2^{-/-}$ (further referred to as $jnk^{-/-}$) mouse embryos (16) in which targeted disruption of the *jnk1* and *jnk2* has been performed simultaneously, and *junAA* immortalized fibroblasts derived from mouse embryos carrying a mutant *c-jun* allele in which the JNK phospho-acceptor serines 63 and 73 are mutated to alanines (17) were a kind gift from Erwin F. Wagner (IMP, Vienna, Austria). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, and antibiotics (100 units/ml penicillin, 50 μ g/ml streptomycin-G and 0.25 mg/ml Fungizone™). Human recombinant TGF- β 1, purchased from R&D Systems Inc. (Minneapolis, MN), referred to as TGF- β throughout the text, was used at a concentration of 10 ng/ml. SP600125, a specific JNK inhibitor (18,19), was purchased from Calbiochem-Novabiochem GmbH (Schwalbach, Germany).

Collagen matrix contraction-Fibroblasts were harvested from monolayer culture with 0.25% trypsin and 1 mM EDTA, following which trypsin was neutralized with 10% FCS-containing medium. Collagen lattices in 60-mm dishes were prepared with 7 ml of a mixture containing 10^6 fibroblasts and 1 mg/ml native type I collagen (Biocoat™, Becton-Dickinson, Bradford, MA) in medium supplemented with 10% FCS. When needed, TGF- β was added to the mixture before polymerization of the collagen matrix. Polymerization of collagen matrices required approximately 60 min. at 37°C. To initiate lattice contraction, freshly polymerized matrices were released from the underlying culture dish with a few gentle taps on the dish.

Wound Closure and Transwell™ Motility Assays-For wound closure assays, confluent cell monolayers were wounded by manually scraping the cells with a pipet tip. Following wounding, wound size was verified to ensure that all wounds were the same width (see

corresponding figures). The cell culture medium was then replaced with fresh medium and wound closure was monitored by microscopy at various times. Transwell™ migration assays were performed utilizing 8- μ m pore, 6.5 mm polycarbonate Transwell™ filters (Falcon, Franklin Lakes, NJ). In some experiments, a type I collagen solution (10 μ g in 100 μ l) was allowed to polymerize in the upper well for 1h at 37°C. Single cell suspensions were seeded onto the upper surface of the filters in medium containing 10% FCS (without or with prior collagen coating) and allowed to migrate through the membrane. After a 16-h incubation period, cells on the upper surface of the filter were wiped off with a cotton swab, and the cells that had migrated to the underside of the filter were fixed, stained with DiffQuick™ (Dade Behring, Düringen, Switzerland), and counted by bright-field microscopy at 200x in six random fields.

Northern Blotting-Total RNA was obtained using an RNeasy kit (Qiagen GmbH, Hilden Germany) and analyzed by Northern hybridization (20 μ g/lane) with ³²P-labeled cDNA probes for *COL1A1* (20), *COL1A2* (21), *COL3A1* (22) and *GAPDH* (23). Hybridization signal was revealed with a phosphorimager (Storm 840, Amersham-Pharmacia, Piscataway, NJ).

Western blotting-Whole cell extracts were prepared in 10 mM Tris, pH 7.4, 1% SDS, 1mM sodium vanadate, treated with Benzon nuclease (Sigma Chemical Co., Saint-Louis, MO) for 5 min. at room temperature, and denatured by heating at 95 °C for 3 min. Protein concentration in each lysate was assayed with a one-step colorimetric method (BIO-RAD protein reagent, BIO-RAD Laboratories, Hercules, CA), and 25 μ g of protein was resolved by SDS-PAGE. After electrophoresis, proteins were transferred to Hybond ECL nitrocellulose filters (Amersham-Pharmacia, Piscataway, NJ). Filters were placed in blocking solution (1x Tris buffered saline, 5% nonfat milk) for 1 h, and immunoblotted with either goat anti-type I collagen (Southern Biotech, Birmingham, AL), rabbit anti-phospho-c-Jun (Upstate

Biotechnologies, Lake Placid, NY), anti-JNK1 (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-phospho-JNK1 (Cell Signaling Technology Inc., Beverly, MA), all at a 1/1000 dilution in 1x Tris buffered saline, 0.1% Tween 20, 5% nonfat milk for 1 h. A mouse anti- β -actin (Sigma) antibody at a 1:10000 dilution in 5% nonfat milk was used as a control. For detection of phosphorylated proteins, non-fat milk was replaced by bovine serum albumin. After incubation, filters were washed and incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa-Cruz, CA) for 1h. Filters were then washed, developed according to chemiluminescence (ECL) protocols (Amersham-Pharmacia) and revealed with a phosphorimager (Storm 840, Amersham-Pharmacia).

Transient cell transfections and electroporation- To determine basal JNK activity, we used a reporter system derived from the mammalian one-hybrid system, consisting of a reporter plasmid, Gal4-Lux, and a *trans*-activator plasmid encoding a chimeric *trans*-activator protein (Gal4BD-c-Jun) consisting of the DNA binding domain of Gal4 (Gal4BD) and the transactivation domain of c-Jun that requires phosphorylation by JNK to fully transactivate Gal4-lux (Stratagene, La Jolla, CA). Transfections were performed using the calcium phosphate/DNA co-precipitation procedure with a commercial assay kit (Promega, Madison, WI). pRSV- β -galactosidase was co-transfected in every experiment to monitor transfection efficiencies. Luciferase activity was determined with a commercial kit (Promega). For high transfection efficiency of a dominant-negative MKK4 (D/N MKK4) expression vector, kind gift from A. Atfi, INSERM U482, Paris, France (24), human dermal fibroblasts were electroporated with a Nucleofector™ (Amaxa GmbH, Koeln, Germany) according to the manufacturer's protocol. Transfection efficiency was estimated by FACS analysis of a co-transfected GFP expression vector to be around 80% (not shown).

RESULTS AND DISCUSSION

We first determined the JNK status under the experimental conditions used in the present study. To this aim, we first measured the respective levels of phospho-c-Jun, phospho-JNK1 and JNK1 in *jnk*^{-/-} and wt mouse fibroblasts, the latter treated or not with SP600125. Results presented in Fig. 1A confirmed the absence of JNK1 expression and the lack of phosphorylation of c-Jun in *jnk*^{-/-} cells. They also demonstrate that SP600125 at a concentration of 20 μ M strongly reduces the basal phosphorylation levels of JNK and c-Jun in wt fibroblasts. These results were further confirmed in the mammalian one-hybrid system described in the “Materials and Methods” section, measuring the transcriptional status of Gal4BD-c-Jun under the various conditions described above (Fig. 1B). We confirm the Western data as, (a) luciferase activity is dramatically lower in *jnk*^{-/-} fibroblasts as compared to their wt counterparts, reflective of an absence of JNK activity in the former cell-type, and (b), SP600125 potently inhibits Gal4-lux activity in wt fibroblasts, consistent with its role as a JNK inhibitor (18,19) acting on detectable basal kinase activity. These results are in agreement with our recent description that overexpression of a dominant-negative form of MKK4, the kinase directly upstream of JNK, reduces phospho-c-Jun-dependent transcription in unstimulated human dermal fibroblasts (15).

Once the JNK status was clearly defined in our experiments, we examined the role its basal activity on fibroblast motility. Initially, we compared the motility of wild-type (wt), *jnk*^{-/-}, and *junAA* immortalized mouse embryo fibroblasts in a wound closure assay. As shown in Fig. 2, wt fibroblasts migrated into the wound area and completely closed the wound within 14 h (upper left panels). On the other hand, in *jnk*^{-/-} fibroblast cultures, the wounds remained open (central left panels). Variations in cell proliferation did not account for the differences

between wt and *jnk*^{-/-} fibroblasts, as wt fibroblasts closed the wound as rapidly in the absence or presence of mitomycin C (4 μ g/ml), a compound that blocks cell division (not shown).

As c-Jun is a key substrate for JNK, *junAA* fibroblasts, in which c-Jun Ser63 and Ser73 have been mutated into alanines so c-Jun can no longer be phosphorylated by JNK, were used to determine whether c-Jun phosphorylation by JNK was important for the effect of basal JNK activity on fibroblast motility. As shown in Fig. 2 (lower panels), *junAA* fibroblasts were identified as poorly motile in the same wound closure assay described above, undistinguishable from *jnk*^{-/-} fibroblasts (central panels). These data indicate that the basal state of c-Jun phosphorylation by JNK is critical for cell migration.

Next, since TGF- β is a known activator of fibroblast motility (25), we tried to determine whether TGF- β would be able to accelerate fibroblast migration in the absence of basal JNK activity. When either *jnk*^{-/-} or *junAA* fibroblasts were treated with TGF- β (Fig. 2, right panels) no significant difference in migration within the wound could be observed as compared to untreated cultures, indicating that TGF- β cannot overcome the impairment in motility induced by the loss of basal JNK activity or by the absence of basal levels of phospho-c-Jun.

The striking difference in cell motility between wt and *jnk*^{-/-} immortalized fibroblasts raised the question whether such phenomenon may be specific for either the knockout or the immortalized phenotype. This led us to determine whether pharmacologic inhibition of JNK activity in either wt immortalized fibroblasts or human dermal fibroblasts would impair their motility in the same wound closure assay. For this purpose, we used SP600125, a specific JNK inhibitor (18,19), at a concentration of 20 μ M, sufficient to inhibit basal JNK activity, as measured by either Western blotting for phospho-JNK or in a modified mammalian two-hybrid system specific for phospho-c-Jun-driven transactivation (see Fig. 1). Under these conditions, wt fibroblasts treated with SP600125 were unable to close a mechanically-

generated wound in a confluent cell layer (Fig. 3A, lower panels), whereas cells treated with the solvent alone did (upper panels). Moreover, consistent with the results obtained using *jnk*^{-/-} fibroblasts, TGF- β was unable to reverse the inhibitory effect of SP600125 in wt fibroblasts. Similar results were obtained with normal human skin fibroblasts (Fig. 3B): DMSO-treated dermal fibroblasts rapidly migrated into the wound, and the latter was completely closed within 20 h (upper panels). On the other hand, SP600125-treated cultures were blocked in their ability to close the wound, even when treated with TGF- β (lower panels).

Together, these experiments identify basal JNK activity as a critical component of the cell machinery allowing fibroblast migration. Interestingly, these results prolong our recent identification of a role for basal JNK activity in maintaining a pool of transcriptionally active, phosphorylated c-Jun protein in fibroblasts, leading to detectable phospho-c-Jun-dependent gene transactivation under unstimulated conditions (15).

We next attempted to determine whether the JNK pathway is involved during collagen gel contraction by fibroblasts, a phenomenon that involves both extracellular matrix remodeling and cell motility (reviewed in (26)). To this aim, we compared the behavior of wt, *jnk*^{-/-}, and *junAA* fibroblasts placed in a free-floating, mechanically unloaded, collagen gels. The kinetics of collagen gel contraction was then recorded over a 7-day period. As shown in Fig. 4, *jnk*^{-/-} and *junAA* fibroblasts were significantly more potent than their wt counterparts to contract collagen gels. Maximal lattice contraction by *jnk*^{-/-} and *junAA* fibroblasts was sub-maximal 2 days after plating, leading to a reduction of about 70% of the original lattice size, whereas gel contraction by wt fibroblasts never exceeded 30%, even over a 15-day period (not shown). No significant changes in cell proliferation within the collagen gels were detected with either cell type over the time course of the experiments, as measured by counting cells following bacterial collagenase digestion of the lattices (not shown). When added immediately after

mixing the cell suspension in the collagen solution, TGF- β significantly accelerated gel contraction by wt, *jnk*^{-/-} and *junAA* fibroblasts. A representative experiment is shown in Fig. 4A. Under such experimental conditions, lattice contraction reached the same extent with either wt fibroblasts or *jnk*^{-/-} and *junAA* fibroblasts, although the maximum was attained slightly later in the case of wt fibroblasts (Fig. 4B). Together, these results demonstrate that basal JNK activity reduces the capacity of fibroblasts to contract collagen gels but does not alter the potentiation of this phenomenon by TGF- β .

Noteworthy, we recently identified JunB, another JNK target, as a potential substitute for c-Jun to mediate antagonistic effects of TNF- α against TGF- β -dependent gene transcription in *junAA* fibroblasts (15). Specifically, we determined that TNF- α efficiently blocks TGF- β signaling in *junAA* fibroblasts, whereas it does not in *jnk*^{-/-} fibroblasts. A dominant-negative mutant form of MKK4 that prevents JNK activation efficiently blocked the effect of TNF- α against TGF- β in *junAA* cells, indicating that this inhibitory mechanism is dependent on JNK function and utilizes a substrate other than c-Jun. By means of antisense approaches, we determined that JunB substitutes for JunAA to mediate the inhibitory activity of TNF- α against Smad signaling in a JNK-dependent manner. Contrasting with the latter results, in the present investigation carried out in the absence of cytokine stimulation (i.e., when only basal JNK activity may be involved), *junAA* and *jnk*^{-/-} fibroblasts both exhibit impaired motility (Fig. 2) and increased contractile activity (Fig. 4), as compared to their wt counterparts. Such identical behavior suggests that no other JNK substrate is able to replace the JunAA mutant in the context of either cell motility or capacity of fibroblasts to contract collagen gels. One plausible explanation would be that basal JunB expression (i.e. not induced by cytokines) in fibroblasts is very low, and is not sufficient to drive detectable JNK-dependent transcriptional effects, whereas c-Jun is expressed at levels sufficient to transmit basal JNK activity. Another possibility may derive from the fact that JunB is a weaker

transcriptional activator than c-Jun (27-29). In the context of TNF- α -induced JunB neosynthesis, the latter relays, at least in part, some of the JNK signals when c-Jun expression, or activation by JNK, is blocked (15).

Mechanistically, the small G protein Rac has been implicated in both cell motility and floating matrix contraction (30). Furthermore, a parallel decrease in both fibroblast motility and their capacity to contract free-floating gels has been correlated to the aging process (31). Specifically, poorly contractile fibroblasts derived from older tissue donors display lower motility than fibroblasts from younger donors, the latter also exhibiting a better capacity to contract free-floating collagen gels. It appears that the link between collagen gel contraction and cell motility is largely circumstantial, and no definitive mechanistic proof has been established thus far to link these two phenomena. Since the results presented above go against such scheme, we tried to establish another assay capable of addressing the issue of whether cell contact to collagen may modify fibroblast motility. For this purpose, we utilized another motility assay based on the Transwell™ system, able to measure the cell migratory potential through a nylon membrane pores in the absence or presence of a collagen coating (see Materials and Methods). As shown in Fig. 5, *jnk*^{-/-} fibroblasts exhibited a much lower capacity to migrate through the Transwell™ membrane than their wt counterparts, which was not influenced, either positively or negatively, by the collagen coating. These data, in full compliance with those obtained using the *in vitro* wound closure assay presented in Figs. 2 and 3, allow us to conclude that basal JNK activity is an important parameter allowing fibroblast motility.

We next examined whether basal JNK activity may influence basal fibrillar collagen gene expression and its upregulation by TGF- β .

As a first attempt to address this hypothesis, confluent wt and *jnk*^{-/-} fibroblast cultures were incubated for 24 h in the absence or presence of TGF- β , after which expression of type I and type III collagen genes was determined by Northern analysis of total RNA. As shown in Fig. 6A, remarkably similar basal steady-state mRNA levels for *COL1A1*, *COL1A2* and *COL3A1* were detected in wt and *jnk*^{-/-} fibroblasts. A 24-h incubation with TGF- β resulted in marked upregulation of the expression of each of these genes, which was identical in its magnitude in both cell types. Type I collagen production, estimated by Western blotting (Fig. 6B), showed identical basal levels in both wt and *jnk*^{-/-} fibroblasts, as well as similar induction in response to TGF- β .

Secondly, human dermal fibroblasts were transfected with either empty or D/N MKK4 expression vectors by means of a Nucleofector™ (see Materials and Methods), following which collagen gene expression was measured by Northern hybridization. Results presented in Fig. 6C demonstrate that D/N MKK4 expression does not modify either basal (*lane 3 versus lane 1*) and TGF- β -induced (*lane 4 versus lane 2*) collagen mRNA steady-state levels. Together with the results obtained using *jnk*^{-/-} fibroblasts, these data demonstrate that basal JNK activity in fibroblasts is not a determinant for either basal fibrillar collagen gene expression or the extent of its activation by TGF- β , contrasting sharply with the critical role played by JNK activation in the ability of inflammatory cytokines to antagonize the upregulation of fibrillar collagen genes by TGF- β (15).

CONCLUSIONS

Cell migration has been considered a key event responsible for collagen gel contraction by fibroblasts under mechanically unloaded conditions. The results presented in this report suggest that a correlation between the two phenomena is only coincidental, as cellular

signaling driven by basal JNK activity differentially modulates these two cellular functions. Specifically, we provide definitive evidence for a critical role for basal JNK activity in allowing fibroblasts to migrate whereas, at the mean time, it severely alters their capacity to contract collagen gels and does not affect fibrillar collagen gene expression.

Our data identify the basal activity of the JNK pathway as highly selective in controlling several fibroblast functions essential for tissue repair, namely cell migration, matrix contraction, and collagen biosynthesis. *In vivo* relevance to wound healing requires further investigations.

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LEGENDS TO FIGURES

FIG. 1. Basal JNK expression and activity in wt and *jnk*^{-/-} mouse embryo fibroblasts; effect of SP600125. *A*, Sub-confluent wt and *jnk*^{-/-} fibroblast cultures were incubated for 24 h in medium containing 10% serum. SP600125 (20 μ M) was added to wt fibroblast cultures 1 h before cell lysis for Western analysis with specific antibodies directed against phospho-c-Jun, phospho-JNK, JNK or β -actin. *B*, Sub-confluent wt or *jnk*^{-/-} fibroblast cultures were transfected with 1 μ g Gal4-lux together with 50 ng Gal4BD-c-Jun and 0.2 μ g of pRSV- β -galactosidase expression vectors. 3 h after transfections, wt cell cultures were treated with either DMSO alone or DMSO containing SP600125 for 20 h, before luciferase activity was determined.

FIG. 2. *jnk*^{-/-} and *junAA* fibroblasts exhibit impaired motility in an *in vitro* wound closure assay. Confluent wt, *jnk*^{-/-} and *junAA* immortalized mouse fibroblast cultures pre-treated or not for 8 h with TGF- β , were wounded with a pipet tip. Following wounding, cell culture medium was replaced with fresh medium, in the absence or presence of TGF- β , and wound closure was monitored by microscopy. A representative micrograph for each condition is shown, taken immediately (0h) or 14 h after wounding. Experiments were repeated at least three times with similar results.

FIG. 3. The JNK inhibitor SP600125 inhibits fibroblast motility. Confluent wt immortalized fibroblast cultures (A) or normal human dermal fibroblast cultures (B) were pre-incubated for 8 h with either DMSO alone or SP600125 in DMSO (20 μ M) before cell layers were wounded and further incubated in the absence or presence of TGF- β , as described in Fig. 2. A representative micrograph for each condition is shown, taken immediately (0h) or 20 h after wounding. Experiments were repeated at least three times with similar results.

FIG. 4. *jnk*^{-/-} and *junAA* fibroblasts exhibit enhanced capacity to contract free-floating collagen lattices. Contraction of free-floating collagen lattices seeded with either wt, *jnk*^{-/-} or *junAA* immortalized mouse fibroblasts (for details, see Materials and Methods) was carried out for the times shown (1d-7d), in the absence (-) or presence (+) of TGF- β . *A*, photographs of a representative experiment. *B*, graphic representation of the experiment shown in *A*: untreated cultures, closed symbols, TGF- β -treated cultures, open symbols. wt fibroblasts, squares, *jnk*^{-/-} fibroblasts, circles; *junAA* fibroblasts, diamonds. Little variation (<15%) was observed in four independent experiments.

FIG. 5. Collagen does not modify the motility of wt and *jnk*^{-/-} fibroblasts. The migration of wt and *jnk*^{-/-} fibroblasts was determined in a Transwell™ assay system, in the absence or presence of native type I collagen (see Materials and Methods). Results are mean \pm S.D. of triplicate samples in a representative experiment.

FIG. 6. Basal JNK activity does not alter fibrillar collagen gene expression levels. *A*, Subconfluent wt and *jnk*^{-/-} fibroblast cultures were incubated for 24 h in medium containing 1% serum, in the absence (-) or presence (+) of TGF- β . Total RNA was analyzed by Northern hybridizations with *COL1A1*, *COL1A2*, *COL3A1* and *GAPDH* probes. A representative autoradiogram is shown. *B*, after incubations, levels of type I collagen and β -actin production were determined by Western analysis of whole cell lysates with specific antibodies. *C*, dermal fibroblasts were transfected with either pRSVe or D/N MKK4 expression vectors using the Nucleofector™ (see Materials and Methods). The next day, the cultures were treated with TGF- β . 24 h later, total RNA was analyzed by Northern blotting with *COL1A2*, *D/N MKK4* and *GAPDH* probes.

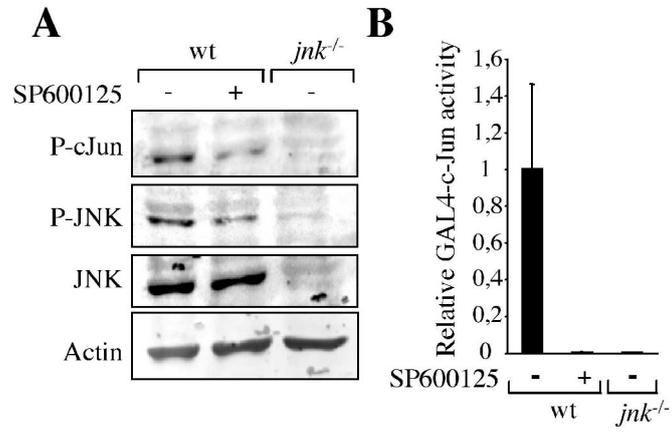


Figure 1

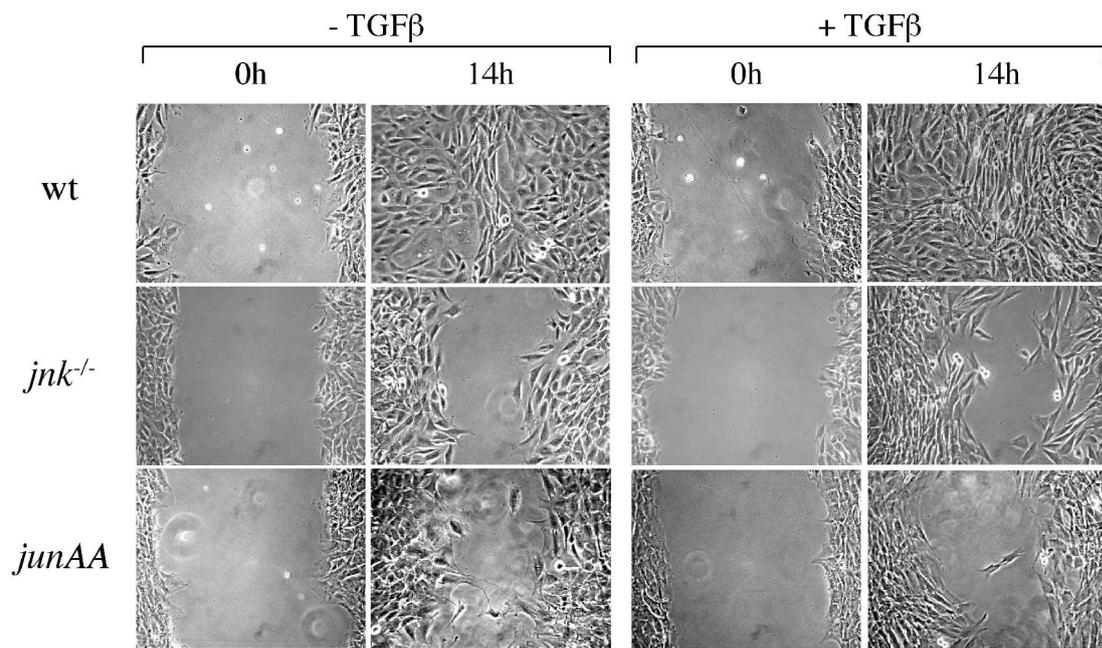


Figure 2

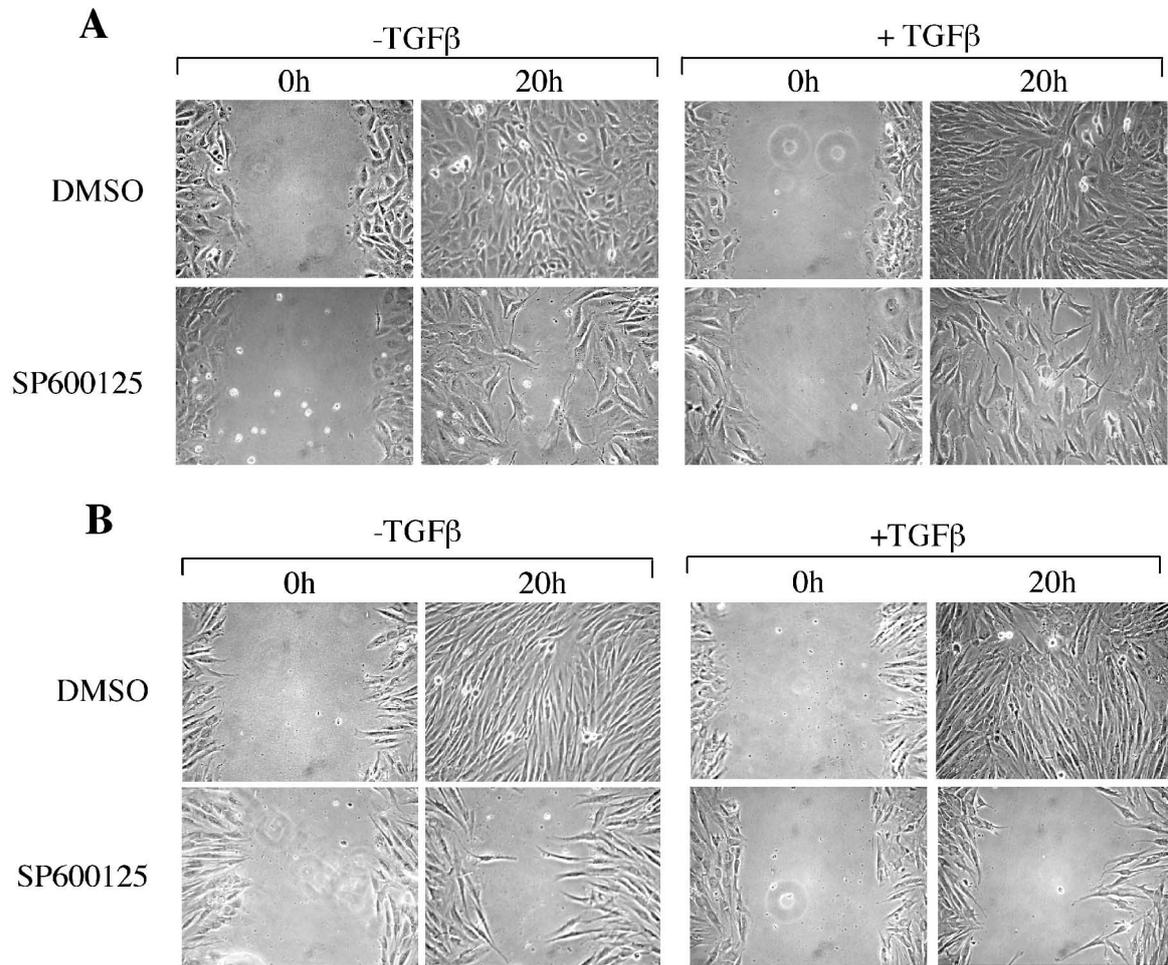


Figure 3

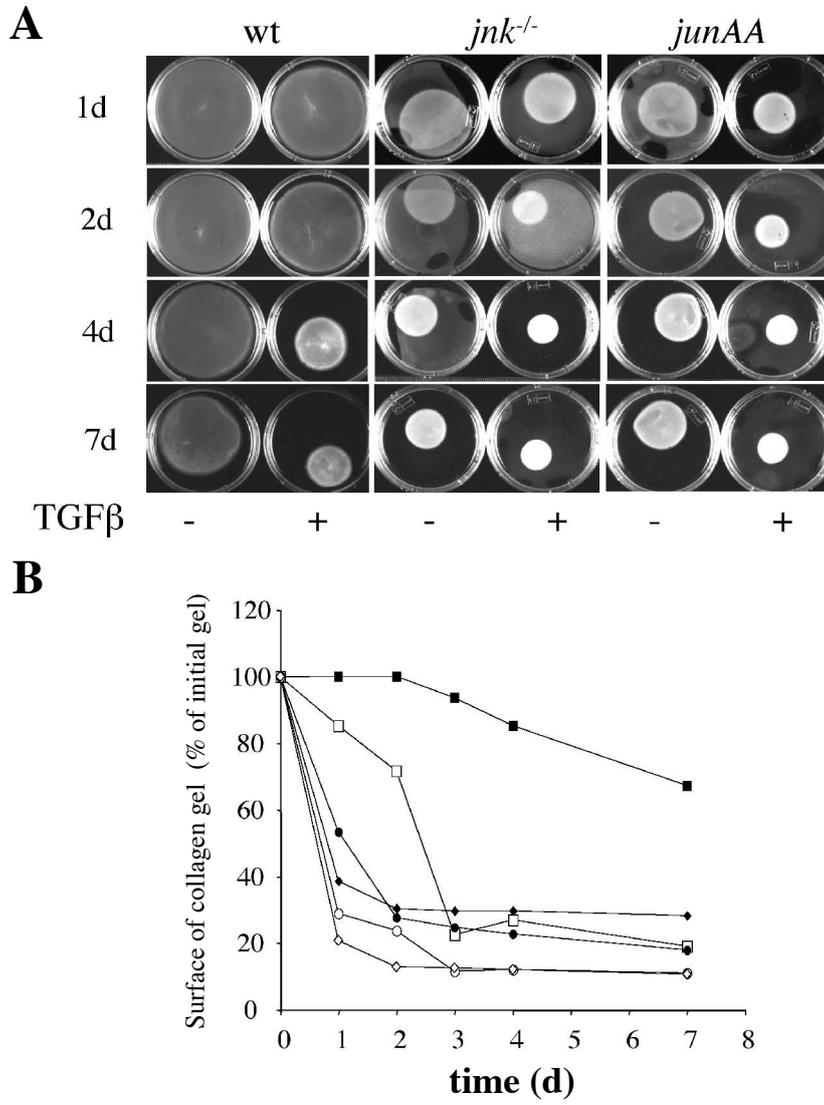


Figure 4

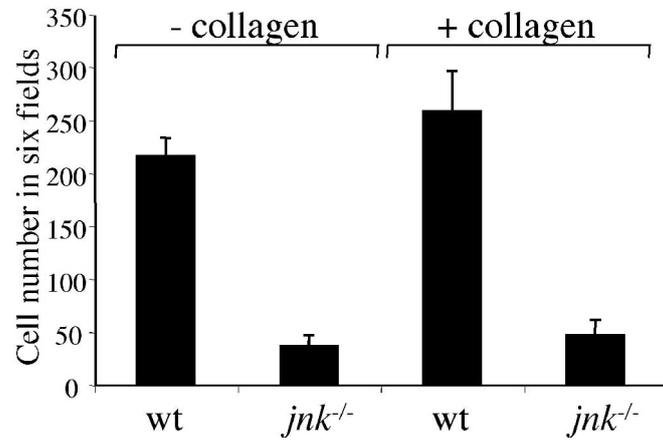


Figure 5

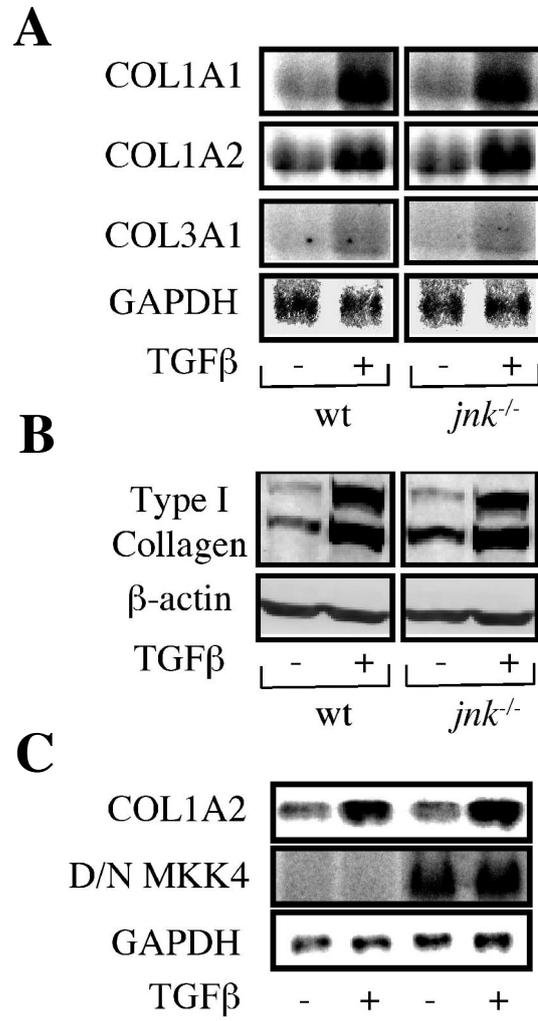


Figure 6