EGF-R Regulates MMP Function in Fibroblasts Through MAPK and AP-1 Pathways

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EGF-R regulates cell proliferation, migration, and invasion in fibroblasts. However, the connection of EGF-R with downstream signaling pathways mediating these responses has remained elusive. Here we provide genetic and biochemical evidence that EGF-R- and AP-1-mediated signals are required for MMP expression and collagen contraction in fibroblasts. In EGF-R (+/−) mouse embryonal fibroblasts, basal and inducible expression of several MMPs, including MMP-2, -3, and -14 is impaired in comparison to wild-type counterparts. The loss of MMP expression is associated with a suppression of EGF-induced Erk and Jnk activities, and AP-1 DNA-binding and transactivation capacities. While inhibition of Jnk mainly prevents EGF-induced phosphorylation of c-Jun, inhibition of Erk pathway suppresses both the expression and phosphorylation of c-Jun and c-Fos proteins. Moreover, the expression of MMP-3 and -14, and collagen contraction is partially prevented by Mek/Erk and Jnk inhibitors. However, Jnk inhibitor also suppresses cell growth independently of EGF-R activity. The central role of AP-1 as a mediator of EGF-R signaling in fibroblasts is emphasized by the finding that expression of a dominant negative c-Jun downregulates the expression of MMP-3. Conversely, expression of a constitutively active Mek1 can induce MMP-3 expression independently of upstream signals. The results indicate that ERK pathway and AP-1 are downstream effectors of the EGF-R-mediated MMP-3 expression and collagen contraction in fibroblasts.


ErbB family of tyrosine kinase receptors are key regulators of cells by engaging growth factors and triggering downstream signaling events that govern cell proliferation, survival, differentiation, and migration (Prenzel et al., 2001; Yarden and Sliwkowski, 2001). The first identified ErbB family member, epidermal growth factor receptor (EGF-R), is an essential tyrosine kinase receptor for both epithelial cells and fibroblasts. Interestingly, fibroblast mobility and proliferation are regulated to a large extent by epidermal growth factor (EGF), transforming growth factor-α (TGF-α), and heparin-binding EGF-like growth factor (HB-EGF), which are ligands for EGF-R (Chen et al., 1994a,b, 1996; Xie et al., 1998). These factors are known to participate in all stages of wound healing (Wells et al., 1998).

Binding of EGF-superfamily growth factors to EGF-R activates four major pathways, that is, the phosphatidylinositol-3 kinase (PI3 kinase), signal transducer and activator of transcription (STAT), phospholipase C-protein kinase C (PLC-PKC), and Ras-mitogen-activated protein kinase (Ras-MAPK) pathways (Prenzel et al., 2001; Yarden and Sliwkowski, 2001). All these pathways have been implicated in growth control and survival. In addition, EGF-R-mediated activation of PLC and MAPKs have been linked to migration and invasion (Chen et al., 1994b; Xie et al., 1998; Cheresh et al., 1999; Glading et al., 2000).

In this study, we have investigated the role of MAPKs in EGF-R-mediated signaling in fibroblasts. Major MAPK pathways consist of extracellular signal-regulated kinases (Erk1/2), c-Jun N-terminal kinases (Jnks) and p38s. Erk-type MAPKs are mainly activated by mitogens and growth factors during cell proliferation and differentiation, whereas Jnks and p38 MAPKs, which belong to the stress-activated protein kinases (SAPKs), are activated in response to inflammatory cytokines, ultraviolet irradiation, heat or osmotic shock (Chang and Karin, 2001). More recently, it has been shown, however, that Jnk and p38 MAPKs also play a role in development, proliferation, differentiation and survival (Davis, 2000; Nebreda and Porras, 2000).

The biological effects of MAPKs are mediated by downstream phosphorylation of substrates, which in the nucleus are often transcription factors. One of the major downstream effectors for MAPKs is the signal inducible transcription factor activator protein-1 (AP-1) (Whitmarsh and Davis, 1996). Originally, AP-1 was characterized as a “TPA-inducible” factor, which regulates...
the transcription of genes containing specific AP-1 recognition sites (TPA responsive elements, TREs) in their promoters (Angel et al., 1987). Subsequent studies showed that AP-1 is a dimeric protein complex composed of different Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, Fra-1, Fra-2, and FosB) subunits. Upon activation Jun homo- or Jun/Fos heterodimers are formed followed by acquisition of TRE-binding activity, which together with other cis-acting elements regulate target gene expression. The MAPK-mediated phosphorylation of AP-1 subunits stimulates their stabilities, DNA-binding affinities and transcriptional activities.

Based on the initial identification of an AP-1 binding site in the promoter of collagenase/MMP-1 gene, and its role in the degradation of extracellular matrix (ECM), AP-1 has been implicated in the regulation of genes involved in matrix remodeling (Westermarck and Kahari, 1999; Ozanne et al., 2000; Angel et al., 2001). More recently, EGF-R-mediated migration and invasion have also been linked to AP-1 transcription factor activity. For example, EGF-induced invasion of both fibroblasts and epithelial cells is AP-1-dependent (Lamb et al., 1997; Malliri et al., 1998). Furthermore, both EGF-R and one of its ligands, HB-EGF, are direct transcriptional targets for AP-1 (Scott et al., 2004). Thus, data are emerging to reveal a functionally significant crosstalk between EGF-R and AP-1. However, the signaling pathways connecting cell surface EGF-R and nuclear AP-1 that are important for matrix remodeling remain to be elucidated.

In this article, we have investigated the proliferation and matrix remodeling of EGF-R deficient fibroblasts and specifically focused on the role of MAPKs and AP-1 in this process. We find that inactivation of EGF-R signaling results in impaired fibroblast proliferation and collagen contraction. Our genetic and biochemical data indicate that collagen contraction of fibroblasts is EGF-R dependent, and that the signals culminating in AP-1 activity and MMP expression are mediated by Erk and Jnk pathways.

Materials and Methods

Cell culture

Mouse embryonal fibroblasts (MEFs) were established from EGF-R (+/−) and wild-type (wt) E17 embryos as previously described (Hogan et al., 1994). The cells were cultured in humidified 5% CO2 atmosphere at 37 °C in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 unit/ml penicillin, and 100 μg/ml streptomycin. Prior to stimulation with 20 ng/ml EGF, 10 nM TPA (Sigma, St Louis, MO) or UV light (30 J/m2), the cells were starved overnight in serum free medium. The cells were harvested, centrifuged and quick-frozen in liquid nitrogen. For analyses of proliferation, the cells were plated at a density of 50,000 cells/60 mm diameter well in duplicates and cultured for 3–7 days. Cell number was determined by trypsinization using Burker chamber and light microscopy. The statistical significance of differences seen in proliferation assays was analyzed using student’s t-test. All P-values were two tailed.

Collagen contraction assay

Collagen gels were prepared using Collagen Type I (BD Biosciences, Bedford, MA). Seven volumes of collagen were mixed with two volumes of fivefold concentrated DMEM and one volume of 0.2 M HEPES (pH7.4), and kept on ice. Cells were mixed gently into neutralized collagen solution before transferred into 24-well plates (50,000 cells/well). Collagen polymerization was initiated by incubating the plates at 37 °C for 30 min. Gels were detached from the well walls and cell culture media containing 10% FBS and appropriate MAPK and EGF-R inhibitors were added into the wells. Contraction process was observed daily. The statistical significance of differences seen in contraction assays was analyzed using student’s t-test. All P-values were two tailed.

RNA isolation, microarray, and Northern analysis

Total RNA was isolated from MEFs by the single step method using Trizol (Invitrogen, Carlsbad, CA). A GEArray Q series mouse ECM and adhesion molecule gene array kit was obtained from SuperArray, Frederick, CA. One microgram RNA was used as a template to generate [γ-32P]dCTP-labeled cDNA probes according to the manufacturer’s instructions. The cDNA probes were denatured and hybridized at 60 °C with the SuperArray membrane, which was washed and exposed to X-ray film. Film was scanned and imported into GEarray analyzer program for further analysis. For Northern blot analysis, 10 μg of RNA was separated on a 1% agarose-formaldehyde gel and transferred to nylon membrane (Hybond-N, Amersham, Piscataway, CA). Filters were hybridized to [γ-32P]dCTP-labeled cDNAs coding for c-Jun (Bohmann et al., 1987), c-Fos (Curran et al., 1982), MMP-2 (Gack et al., 1994), MMP-3 (Gack et al., 1994), MMP-14 (Madlenor et al., 1998), and GAPDH (Fort et al., 1985) cDNAs. Hybridizations and washing conditions were performed according to the instructions of the manufacturer.

Zymogram assay

Analyses for gelatinase activity was carried out as previously described (Miettinen et al., 2000). PAGE gels (10%) containing 2 mg/ml gelatin (Sigma) were cast. Conditioned cell culture media was applied to the gel in non-reducing 4:1 Laemmli sample buffer. After electrophoresis, the gels were rinsed in washing buffer (2.5% Triton X-100, 50 mM Tris-HCl pH 7.6, 5 mM CaCl2, 1 μM ZnCl2) for 2 x 15 min at RT. Next, the gels were washed in reaction buffer (50 mM Tris-HCl pH 7.6, 5 mM CaCl2, 1 μM ZnCl2) for 5 min at RT and incubated in reaction buffer overnight at 37 °C. The gels were stained with Coomassie brilliant blue and destained with solution containing methanol:acetic acid:water (2: 1: 7 by volume).

Western analysis

MEFs were lysed directly in sodium dodecyl sulfate (SDS) sample buffer and sonicated with a microtipped sonicator. Equal amount of protein was separated on a 10% SDS–PAGE gel, and transferred onto nitrocellulose membranes (BioRad, Hercules, CA) by electroblotting. Immunoblotting was performed using polyclonal antibodies against MAP kinases (phospho-Erk, Promega, Madison, WI; phospho-p38, Cell Signaling Technology, Beverly, MA; p38, Erk1/2, and Jnk1/2, Santa Cruz Biotechnology Inc., Santa Cruz, CA), and AP-1 proteins (phospho-c-Jun, Cell Signaling Technology; c-Jun, and c-Fos, Santa Cruz). Horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson Laboratories, West Grove, PA. The blots were developed using an enhanced chemiluminescence (Super Signal, Pierce, Rockford, IL).

Gel mobility-shift assay

The cells were harvested, centrifuged and quick-frozen in liquid nitrogen. Cell pellets were homogenized in two volumes of buffer containing 20 mM HEPES pH 7.9, 0.04 M NaCl, 25% (w/v) glycerol, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM diethiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 2.5 μM leupeptin, 25 μM (l-glycerophosphate, and 0.1 mM Na2VO3 (Sigma). Amounts of soluble protein were measured using BCA Protein Assay Kit (Pierce). To assay AP-1 DNA-binding activity, cell extracts were incubated for 20 min at room temperature (RT) in a 20 μl reaction buffer containing 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, 5% (v/v) glycerol, 0.5 mM DTT, 0.5 mM PMSF, 400 μg/ml poly (dl-DC), 400 μg/ml BSA, and 0.1 ng [γ-32P]ATP-labeled oligonucleotide probe containing the TRE-element. Protein–DNA complexes were resolved on a 4% non-denaturing polyacrylamide gel containing 0.5 x TBE and visualized by autoradiography.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) was performed as previously described (Kang et al., 2002). Briefly, wt MEFs were fixed with
formaldehyde and sonicated. Lysates were preincubated with protein A Sepharose and subjected to immunoprecipitation overnight at 4 °C with normal rabbit IgG or antibodies against c-Jun and c-Fos (Cell Signalling Technology and Santa Cruz, respectively). Precipitates were washed several times and eluted from beads with elution buffer (1% SDS, 0.1 M NaHCO3). Crosslinking was reverted by adding NaCl to concentration of 300 mM and heating at 67 °C for 4 h. The samples were precipitated with ethanol overnight at -20 °C. After centrifugation, DNA was suspended to TE buffer, recovered using NucleoSpin Extract II purification system (Macherey-Nagel) and analyzed for AP-1 promoter sequence in MMP-3 gene using PCR.

PCR analysis of immunoprecipitated DNA

PCR was performed on ChIP products for 30 cycles using DyNAzyme II polymerase (Finnzymes, Espoo, Finland). Control reactions with mouse genomic DNA were always carried out along the immunoprecipitated samples. The following primers for MMP-3 gene fragment were used: (-189/+97) S'-TGCCCGAGTTCCTCTTTTG-3' and S'-CGGAAGACCGCTTATTTCA-3'. The PCR products were fractionated on agarose gels, stained with ethidium bromide and analyzed using Alphalmag™ 2200 Documentation and Analysis System (Alpha Innotech Corp., San Leandro, CA).

In vitro kinase assays

Cells were washed with PBS and solubilized in lysis buffer containing 25 mM HEPES–NaOH pH 7.5, 150 mM NaCl, 1% Triton X-100, 100 mM glycerol, 5 mM EDTA, 5 mM EGTA, 25 mM β-glycerophosphate, 0.1 mM Na2VO4. Jnk was immunoprecipitated using polyclonal Jnk antibody (Santa Cruz) for 1 h at 4 °C. Immunocomplexes were coupled to protein-A-Sepharose beads for 1 h and washed several times with dilution buffer (25 mM HEPES pH 7.5, 5 mM EDTA, 5 mM EGTA, 25 mM β-glycerophosphate, 0.1 mM Na2VO4). Kinase reactions were performed in kinase buffer (50 mM HEPES pH 7.5, 10 mM MgCl2, 1 mM DTT, 25 mM β-glycerophosphate, 1 mM Na2VO4, 100 μM ATP) for 20 min at 30 °C using GST-c-Jun protein (amino acids 5–105) as a substrate. The phosphorylated c-Jun proteins were analyzed on a 10% SDS–PAGE and immunoblotting using an antibody against c-Jun phosphorylated on serine 73 (Cell Signalling Technology).

Plasmids, transfections, and luciferase assays

For transactivation studies, duplicates of 60-mm diameter plates containing 50,000 cells were transfected with the luciferase reporter constructs for collagenase I and mutated collagenase I promoters (AP-1/Ets-; intact AP-1 and ETS-binding sites, and AP-1 Ets-; mutated AP-1 site but functional ETS-binding site) (Treier et al., 1995) using the Fugene-6 reagent (Roche, Mannheim, Germany). After 18 h the cells were stimulated with 20 ng/ml EGF or 10 nM TPA for additional 6 h. The activity of collagenase reporter was normalized to the protein concentration. Luciferase assays were performed according to the manufacturer’s instructions (Promega).

Adenovirus infection of MEF cells

Wt or EGF-R (+/-) MEFs were cultured on 10 cm plates in DMEM containing 1% FBS and infected with recombinant adenoviruses for dominant negative c-Jun (AdTAM67) (Auer et al., 1998) or catalytically active Mek1 (AdMek1ca) (Foschi et al., 1997) in a total volume of 3 ml containing 1% FBS and infected with recombinant adenoviruses for additional 6 h with or without EGF (20 ng/ml).

Results

Collagen contraction is EGF-R-dependent in mouse embryonal fibroblasts

To determine the function of EGF-R in fibroblasts, mouse embryonal fibroblasts (MEFs) were isolated from embryos with a targeted null mutation in the EGF-R gene (Miettinen et al., 1995). In comparison to the wt fibroblasts, the proliferation rates and saturation densities of the EGF-R (+/-) MEFs at early passages were reduced (Fig. 1A). However, when the growth of the cells at later passages was examined, no significant differences between wt and EGF-R (+/-) fibroblasts were detected (Fig. 1A). Furthermore, no differences in saturation density, apoptosis, morphology, and stress fiber formation were observed between the genotypes (data not shown).

Fig. 1. Proliferation and collagen contraction are EGF-R dependent in mouse embryonal fibroblasts. A: Proliferation is impaired in EGF-R-deficient primary MEFs. EGF-R wt and (+/-) MEFs from passages 3 and 13 were cultured in the presence of FBS. After 3, 5, and 7 days the cells were counted. The data are the mean ± SE of two parallel samples. B: Collagen contraction is EGF-R dependent. Wt and EGF-R (+/-) MEFs were cultured inside collagen gels for 5 days together with FBS, EGF-R (ZD), and MMP (BB) inhibitors, as indicated. The percentage of maximal diameter (100%) is shown. The data are the mean ± SD of three separate samples. Statistically significant differences from wt cells are indicated as follows: *P < 0.01. The experiment was repeated three times with similar results. C: The specific EGF-R inhibitor prevents Erk phosphorylation. Whole-cell extracts were isolated from control, EGF-, FBS-, and EGF-R inhibitor (ZD)-treated wt MEFs, as indicated. Equal amounts of protein were subjected to SDS–PAGE and immunoblotting using antibodies against phospho-Erk and Erk.
the EGF-R (−/−) cells demonstrated contraction of only 24%. The findings that EGF-R inhibitor prevented contraction of wt and EGF-R (−/−) MEFs by 18% and 19%, respectively, confirmed that collagen contraction of fibroblasts was primarily EGF-R-dependent.

**MMP expression and activity are impaired in EGF-R deficient fibroblasts**

Impaired collagen contraction of EGF-R-deficient MEFs suggested a defect in matrix remodeling. We next analyzed the involvement of MMPs in this process. MMP inhibitor batimastat prevented collagen contraction of the wt MEFs, indicating that the capacity of fibroblasts to contract collagen requires MMP activity (Fig. 1B). To identify the EGF-R-dependent ECM molecules, we compared RNA expression of wt and EGF-R (−/−) MEFs using ECM and adhesion pathways-focused microarrays. Microarray screen identified a significant downregulation in the expression of several MMPs in the EGF-R-deficient cells. The most obvious suppression was observed in MMP-2, -9, -11, and -13, and -17 mRNA levels (data not shown). Tissue inhibitor of metalloproteinases-1 (TIMP-1) was also downregulated, while TIMP-2 levels were upregulated. Furthermore, no suppression of ECM components of collagen (coll I and collagen IV, fibronectin (FN), and laminin (LM)) was observed.

Decreased MMP levels were investigated in more detail by Northern blotting (Fig. 2A). While basal MMP-9 mRNA levels were too low for detection (data not shown), MMP-2, -3, and -14 mRNA levels were constitutively expressed in wt MEFs. To enhance EGF-R activity, we used a specific EGF-R ligand, EGF, in subsequent studies. Stimulation with EGF further increased the MMP-3 mRNA levels in wt MEFs. In contrast, the mRNA expression of all MMPs was only barely detectable in EGF-R (−/−) MEFs. Consistent with the low MMP-2 mRNA expression, the activity of MMP-2, as measured by zymogram assay, was significantly reduced in the EGF-R (−/−) MEFs (Fig. 2B).

**MMP expression correlates with AP-1 activity**

Based on the identification of the AP-1 binding site in the collagenase promoter (Angel et al., 1987), AP-1 has been implicated as a direct regulator of MMP activity. Subsequent analyses have identified putative AP-1 binding sites on several MMP promoters (Benbow and Brinckerhoff, 1997). To determine whether EGF-R activity was required for AP-1 activity, we analyzed EGF-induced expression of the major AP-1 subunits, c-jun and c-fos in wt and EGF-R (−/−) MEFs by Northern blotting. As shown in Figure 3A, exposure of the wt MEFs to EGF caused a rapid induction of c-jun and c-fos mRNAs. In contrast, no induction was observed in the EGF-R (−/−) MEFs (Fig. 3A). Correspondingly, a prominent induction of c-Fos protein was seen only in the wt cells (Fig. 3B). While c-Jun was expressed both in the wt and EGF-R (−/−) fibroblasts, the EGF-dependent increase in the expression and phosphorylation of c-Jun was detected only in the wt cells (Fig. 3B).

To test whether the increased expression of c-Jun and c-Fos was followed by acquisition of AP-1 DNA-binding activity, we performed gel mobility shift assays. The seven base pair

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**Fig. 2.** MMP expression and activity are impaired in EGF-R deficient MEFs. A: Expression of MMP mRNAs in wt and EGF-R (−/−) MEFs. Total RNA was isolated from control and EGF-treated wt and EGF-R (−/−) MEFs at indicated time periods. Equal amount of RNA was analyzed by Northern blotting using 32P-labeled cDNA probes for MMP-3, -14, and -2. GAPDH was used as a loading control. B: Gelatinase activity in wt and EGF-R (−/−) MEFs. Conditioned media from wt and EGF-R (−/−) MEFs cultured in serum free conditions for 24 h was analyzed by gelatin zymography.
consensus sequence TGAGTCA, a TRE, was used as AP-1-binding site. Consistent with the expression data seen in Figure 3, we observed a basal AP-1 DNA-binding activity in the untreated wt MEFs, whereas the binding was barely detectable in the EGF-R (−/−) cells (Fig. 4A). In addition, the basal AP-1 DNA-binding activity was increased in response to EGF only in the wt cells.

To examine whether suppression of AP-1 expression and DNA-binding activity had functional consequences for transcription in the EGF-R deficient MEFs, we compared AP-1 promoter activity in the wt and EGF-R (−/−) MEFs. The cells were transfected with a construct containing a luciferase gene under the control of collagenase 1 (MMP-1) promoter, which includes AP-1 and Ets binding sites, exposed to EGF or TPA, and after 6 h analyzed for luciferase activities. As shown in Figure 4B, basal collagenase promoter activity was lower in the EGF-R (−/−) MEFs, as compared to the wt cells. The promoter activity was slightly (20–30%) enhanced by EGF and TPA in the wt cells. Mutating the AP-1 binding site in the promoter suppressed both the basal and inducible promoter activity of the wt MEFs to about 50%, indicating that both AP-1 and Ets contribute to collagenase promoter activity in MEFs.

Having demonstrated that AP-1 activity is induced in wt MEFs along with MMP-3 expression, we assessed whether AP-1 proteins c-Jun and c-Fos are recruited to the endogenous MMP-3 promoter. Chromatin immunoprecipitation (ChIP) assays were performed on wt MEFs stimulated with EGF (Fig. 4C). Treatment of cells with EGF enhanced binding of both c-Jun and c-Fos to MMP-3 promoter, indicating that MMP-3 is a direct AP-1 target in MEFs. This association was specific, as non-specific immunoglobulins did not immunoprecipitate the promoter.

MMP and AP-1 activities are MAPK dependent

We next investigated which downstream pathway the EGF-R signaling uses for upregulating the AP-1 and MMP expression. The most likely transducers are MAP kinase pathways culminating in the activity of Erk1/2, Jnk1/2, and p38. Hence, Erk, Jnk, and p38 activities were examined by immunocomplex kinase assays or Western analyses using antibodies against the phosphorylated and thereby activated forms of these proteins. EGF rapidly phosphorylated EGF-R on tyrosine 1173 in wt MEFs, followed by induction of Erk activity (Fig. 5A). Activation of Erks 1 and 2 was observed after 10 min of EGF treatment and it remained elevated for 3 h. In comparison, the activity of p38 was barely detectable. Jnk was constitutively active, and the basal Jnk activity was further increased upon exposure of the cells to EGF (Fig. 5B). The kinetics and the amplitude of the Erk and Jnk pathways were compatible with the induction of MMP-3 activities. In contrast, induction of Erk, Jnk, and p38 activities was impaired in response to EGF in the EGF-R (−/−) MEFs. However, TPA and UV could stimulate the MAPK kinase activities in the EGF-R (−/−) MEFs, indicating that the suppression was EGF-R specific in both genotypes (Fig. 5). EGF had no effect on the expression levels of Erk1/2, p38, and Jnk1/2 in either cell type.

EGF-R mediated upregulation of MMP activity involves MAPK and AP-1 signaling

To gain further insight into the role of Erk, Jnk, and p38 pathways in the EGF-R-dependent regulation of MMP expression, we used specific kinase inhibitors. wt MEFs were cultured without (Fig. 6A) or with EGF (Fig. 6B) and EGF-R (ZD1839), Mek (PD98059), p38 (SB203580), and Jnk (SP600125) inhibitors, and analyzed for the expression of MMP-2 mRNA levels. Basal MMP-14 expression was also dependent on EGF-R and Erk signaling. p38 kinase inhibitor did not have any effect on the expression of MMPs, and none of the inhibitors influenced the expression of MMP-2 mRNA or MMP-2 activity. Furthermore, proteolytic MMP-2 activity was not impaired by kinase inhibitors (data not shown). Basis for the impaired MMP-2 expression in the EGF-R (−/−) MEFs is unclear and subject for further investigation. In conclusion, the results indicate that Erk- and Jnk-pathways are required for basal and inducible expression of MMP-3 and -14, whereas neither EGF-R nor MAPK activities are essential for the expression of MMP-2 in MEFs.
Next, we investigated the ability of the Erk, Jnk, and p38 inhibitors to prevent the collagen contraction of MEFs. Addition of Mek and Jnk inhibitors onto wt MEFs partially prevented, whereas p38 inhibitor did not have a significant influence on gel contraction (Fig. 6C). Mek inhibitor did not influence cell numbers (Fig. 6D). In contrast, Jnk inhibitor also suppressed proliferation, suggesting that the suppression of collagen contraction is at least partially caused by decreased cell numbers in wt cells. In comparison, none of the MAPK inhibitors significantly enhanced the contraction defect in EGF-R (-/-) cells. However, Jnk inhibitor had a negative effect on proliferation. Together with the data shown in Figure 1, the results provide evidence that EGF-R-dependent activation of Erk pathway is required for collagen contraction in MEFs, whereas Jnk also regulates proliferation independently of EGF-R.

To address the relationship between MAPK and AP-1 activity in EGF-R-dependent signaling, MEFs were treated with EGF in the presence of EGF-R, Mek, p38, and Jnk inhibitors, and analyzed for c-Jun and c-Fos expression. Consistent with the finding that c-Jun and c-Fos expression was downregulated in the EGF-R deficient MEFs (Fig. 3), EGF-R inhibitor prevented the induction of mRNA levels in response to EGF (Fig. 7A). c-Fos mRNA levels were also dependent on Erk activity, whereas no clear inhibition of c-jun mRNA levels was observed. However, Mek inhibitor decreased c-Jun protein levels and phosphorylation at serine 73, suggesting that c-Jun stability requires Erk activity (Fig. 7B). Likewise, Jnk inhibitor suppressed EGF-induced c-Jun expression and phosphorylation but failed to decrease c-jun and c-fos mRNA levels. In contrast to Mek and Jnk inhibitors, p38 kinase inhibitor did not inhibit but rather stimulated the expression of c-Fos and c-Jun.

If AP-1 was an essential downstream component of the EGF-R-dependent MMP upregulation in fibroblasts, a dominant negative form of AP-1 would be expected to interfere with this process. Thus, wt MEFs were infected with an adenovirus producing TAM67, a truncated, transactivation deficient form of c-Jun, and after 24 h analyzed for the expression of MMP-3 mRNA levels. As shown in Figure 7C, expression of TAM67 in wt MEFs caused a marked inhibition of both basal and EGF-induced MMP-3 mRNA expression. Conversely, expression of activated Mek1 could partially rescue MMP-3 expression in EGF-R (-/-) MEFs (Fig. 7C). The finding is consistent with previous data showing that activated Mek1 stimulates ERK activity and MMP-3 expression in human skin fibroblasts (Reunanen et al., 2002). These results indicate that EGF-R-dependent MMP-3 expression in MEFs is mediated by Mek1 and AP-1 activities.

Discussion
Previous analyses have indicated an indispensable role for EGF-R in proliferation and differentiation of epithelial cells (Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995). However, as tissue morphogenesis is dependent on constant crosstalk between developing epithelia and
surrounding stroma, it is important to address how the regulatory signaling pathways contribute to outcome in both cell types. In this study, we have investigated how EGF-R-mediated signals affect cellular responses in fibroblasts by using MEFs with a targeted null mutation in EGF-R. Our data demonstrate that a proliferation defect, which is observed in primary EGF-R-deficient cells, is lost at later cell passages, suggesting that the cells acquire additional mutations that can overcome the dependence of EGF-R ligands. In agreement with this, wound-induced epithelial proliferation of EGF-R(-/-) skin is impaired at the early stages of healing, but improves at later stages conceivably through other mechanisms (Repertinger et al., 2004). We have currently no information available to show what the compensatory signaling pathways are. Thus far, the findings in Figure 5 exclude the possibilities that certain EGF-R-independent pathways, which ultimately stimulate Erk, Jnk, and p38 activities are involved. However, MMP-3 expression and collagen contraction are impaired also at later passages. The defects in MMP-3 expression are accompanied with suppression of MAPK and AP-1 activities. Thus, MAPK and AP-1 activities are required for EGF-R-dependent ECM remodeling, but not for proliferation in fibroblasts. Consistently, targeted disruption of the c-fos and c-jun gene expression in fibroblasts causes impaired expression of MMP-3 without a defect in proliferation (Hu et al., 1994; McDonnell et al., 1990).

Degradation of ECM, which is a necessary step in tissue remodeling processes, such as wound healing and embryonal development has been attributed to proteolytic activity of MMPs (Ravanti and Kahari, 2000; Sternlicht and Werb, 2001; Mott and Werb, 2004). However, the role of EGF-R-mediated signals in regulating matrix degradation has not been completely unraveled. Thus far, the most compelling evidence that MMPs are downstream effectors for EGF-R activity has been provided by studies analyzing embryonal development in EGF-R null mice. For example, EGF-R inactivation has been shown to impair MMP activity during pancreatic and craniofacial development (Miettinen et al., 1999, 2000). Likewise, EGF-R has been found to control lung branching morphogenesis by regulating MMP-14 expression, which in turn is necessary for activation of MMP-2 (Holmbeck et al., 1999; Kheradmand et al., 2002). Our data are in agreement with these studies, and further demonstrate that EGF-R- and MMP-dependent collagen contraction in fibroblasts requires MAPK and AP-1 activity. Although it is currently not clear if MAPK and AP-1 are required for the developmentally specific responses to EGF-R in vivo,
our data provide insight into the mode of action of EGF-R in fibroblasts. In this context, it is important to note that in certain developmental situations, such as mammary ductal morphogenesis, stromal EGF-R is required for epithelial development, whereas epithelial EGF-R is dispensable (Wiesen et al., 1999; Sternlicht et al., 2005). The finding that the lack of EGF-R signaling in uterine and vaginal stroma, but not in epithelia, accounted for organ hypoplasia in the female genital tract (Hom et al., 1998) further suggest that EGF-R signaling in stromal cells not only contribute to ECM remodeling but also play a growth regulatory role.

Wound healing is another remodeling process, in which properly controlled stromal-epithelial interactions are crucial for outcome (Martin, 1997; Singer and Clark, 1999). For correct healing, epithelial cells must respond to cytokines and growth factors and migrate to close the wound. In EGF-R null mice, early proliferation and migration of keratinocytes are impaired resulting in delayed wound healing (Repertinger et al., 2004). Although wound re-epithelialization is eventually completed in the absence of EGF-R, the data suggest that EGF-R-dependent signals contribute to production of growth factors during early stages of wound healing. Stromal fibroblasts are responsible for secreting cytokines and growth factors that stimulate epithelial cell proliferation and migration. Fibroblasts are also required for matrix deposition, remodeling, and wound contraction, which are widely influenced by MMP activity (Ravanti and Kahari, 2000; Vu and Werb, 2000). Our results show that expression of MMP-3 and -14 in fibroblasts, and subsequent collagen contraction is EGF-R-dependent. MMP-2 expression was also downregulated in EGF-R deficient cells. However, the small-molecular inhibitor of EGF-R, ZD1839, which inhibits ATP binding within the tyrosine-kinase domain of the EGF-R, could not suppress the expression or the proteolytic activity of MMP-2 in wt MEFs. The findings also exclude the possibility that MMP-2 activity is involved in collagen contraction. As proMMP-2 is proteolytically cleaved by MMP-14 to yield active MMP-2 (Holmbeck et al., 1999), the data further suggest that MMP-14 activity may neither be relevant for collagen contraction in fibroblasts. Thus, the most likely candidate for mediating EGF-R-dependent collagen contraction in fibroblasts is MMP-3. Consistent with this, the absence of MMP-3 has been shown to cause impaired collagen and wound contraction capacities (Bullard et al., 1999a,b), whereas no such defect has been reported for MMP-2 and -14 deficient cells. The importance of MAPK/AP-1 pathway in the regulation of proliferative signals in wound healing is well established (Angel and Karin, 1991). Wounding activates several growth factors and cytokines, which stimulate AP-1 activity through MAP kinases. Conversely, number of cytokines, including fibroblast-derived KGF and GM-CSF, whose function in epithelial-mesenchymal crosstalk during wound closure is critical, are regulated by AP-1 proteins c-Jun and JunB (Szabowski et al., 2000). In addition to proliferation, AP-1 has been suggested to have a functional role in tissue remodeling during wound repair, since several genes encoding ECM proteins, MMPs and their inhibitors contain AP-1 regulatory elements, and are induced during wound healing (Mauviel et al., 1996; Benbow and Brinckerhoff, 1997; Jaakkola et al., 1998). However, a precise role of AP-1 under these conditions awaits further clarification. In this study, we have found a connection between EGF-R, MAPKs and AP-1 in fibroblasts. This pathway is indispensable for proliferation only at early passages, suggesting that EGF-R-independent signaling mechanisms overcome the proliferation defect. However, the pathway is essential for ECM remodeling. Together with previous studies in c-Fos- and MMP-3-deficient MEFs (Bullard et al., 1999b), and rat fibroblasts modulated for the expression of c-Jun and c-Fos (McDonnell et al., 1990), our data provide a link between EGF-R activation, MMP expression and wound contraction.

Literature Cited


