The Angiogenic Factor Cyr61 Activates a Genetic Program for Wound Healing in Human Skin Fibroblasts*

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Cyr61 is a heparin-binding, extracellular matrix-associated protein of the CCN family, which also includes connective tissue growth factor, Nov, WISP-1, WISP-2, and WISP-3. Cyr61 is capable of multiple functions, including induction of angiogenesis in vivo. Purified Cyr61 mediates cell adhesion and induces adhesive signaling, stimulates cell migration, enhances cell proliferation, and promotes cell survival in both fibroblasts and endothelial cells. In this study, we have used cDNA array hybridization to identify genes regulated by Cyr61 in primary human skin fibroblasts. The Cyr61-regulated genes fall into several groups known to participate in processes important for cutaneous wound healing, including: 1) angiogenesis and lymphogenesis (VEGF-A and VEGF-C); 2) inflammation (interleukin-1β); 3) extracellular matrix remodeling (MMP1, MMP3, TIMP1, uPA, and PAI-1); and 4) cell-matrix interactions (Col1a1, Col1a2, and integrins αv and α3). Cyr61-mediated gene expression requires heparin binding activity of Cyr61, cellular de novo transcription, and protein synthesis and is largely dependent on the activation of p42/p44 MAPKs. Cyr61 regulates gene expression not only in serum-free medium but also in fibroblasts cultured on various matrix proteins or in the presence of 10% serum. These effects of Cyr61 can be sustained for at least 5 days, consistent with the time course of wound healing in vivo. Interestingly, Cyr61 can interact with transforming growth factor-β1 to regulate expression of specific genes in an antagonistic, additive, or synergistic manner. Furthermore, we show that the Cyr61 gene is highly induced in dermal fibroblasts of granulation tissue during cutaneous wound repair. Together, these results show that Cyr61 is inducibly expressed in granulation tissues after wounding and that Cyr61 activates a genetic program for wound repair in skin fibroblasts. We propose a model in which Cyr61 integrates its activities on endothelial cells, fibroblasts, and macrophages to regulate the processes of angiogenesis, inflammation, and matrix remodeling in the context of cutaneous wound healing.

Cyr61 is a member of the CCN protein family, which also includes CTGF, Nov, WISP-1, WISP-2, and WISP-3 (1, 2). These vertebrate-specific proteins are characterized by a four-domain modular structure that share sequence similarities to insulin-like growth factor binding proteins, von Willebrand factor, thrombospondin, and growth factor cysteine knots (1, 3, 4). The expression of Cyr61 is associated with the cardiovascular and skeletal systems during embryonic development (5). Consistent with this observation, Cyr61 can act as both an angiogenic inducer and a chondrogenesis differentiation factor. Purified Cyr61 supports endothelial cell adhesion and induces cell migration through integrin αvβ3, enhances growth factor-induced mitogenesis, and induces neovascularization in rat cornea (6–8). As an ECM-associated protein, Cyr61 supports cell adhesion, migration, and proliferation in fibroblasts (9, 10). Overexpression of Cyr61 in human tumor cells enhances their tumorigenicity in immunodeficient mice, increasing tumor size and vascularization (7, 11). In addition, Cyr61 enhances chondrogenic differentiation of mouse limb bud mesenchymal cells in micromass cultures, suggesting a role in skeletal development (12). Cyr61 is a ligand of multiple integrin receptors, which mediate some of its activities in different cell types (6, 9, 10, 13). In fibroblasts, Cyr61 induces cell adhesion and adhesive signaling through integrin αvβ3 and heparan sulfate proteoglycans (9, 14), cell migration through integrin αvβ5 and cell proliferation through integrin αvβ3 (10).

Although a number of activities have been documented for Cyr61 as described above (7–10, 14), information on its effects on gene expression is still scant. Cyr61 was shown to elevate type II collagen expression in mesenchymal cells undergoing chondrogenic differentiation (12), whereas fibroblasts adhered to Cyr61 express high levels of MMP1 (collagenase-1) and MMP3 (stromelysin-1) (14). To understand its function fully, we used cDNA microarray hybridization to identify genes regulated by purified Cyr61 in human dermal fibroblasts. Interestingly, the Cyr61-regulated genes thus identified encode proteins that control angiogenesis, inflammation, ECM remodeling, and cell-ECM interaction, all of which are processes vital for cutaneous wound healing (15–17). The notion that Cyr61 is important in wound repair is further supported by the finding that Cyr61 expression is highly induced and tightly regulated in granulation tissue during cutaneous wound healing. Furthermore, Cyr61 regulates gene expression in the presence of other ECM molecules or serum growth factors and its effect can last for at least 5 days, consistent with the tissue environment in which healing occurs and the time...
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MATERIALS AND METHODS

Cell Culture, Proteins, Antibodies, and Reagents—Normal human fibroblasts (1064SK) derived from skin biopsy of healthy newborn were obtained from the American Type Culture Collection (CRL-2076). The cells were maintained in Iscove’s modified Dulbecco’s medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum, (Intergen, Purchase, NY) at 37 °C with 5% CO2, and used before passage 8. Both wild-type and mutant Cyr61 (Cyr61DM) proteins were produced in a baculovirus expression system using SF9 cells and purified from serum-free insect cell conditioned medium on Sepharose-S columns as described (8, 9). FN, vitronectin, laminin, and type I collagen were purchased from Collaborative Biomedical (Bedford, MA). BSA, heparin (sodium salt, from porcine intestinal mucosa), cycloheximide, 5,6-dichlorobenzimidazole riboside, X-gal, Harris hematoxylin, and eosin-Y solutions were from Sigma. PD98059 was purchased from Calbiochem-Novabiochem Corp. (San Diego, CA), as were monoclonal antibodies against human VEGF-A, VEGF-C, integrin β1, MMP3, and PAI-1 were generated by reverse transcription followed by polymerase chain reaction. First strand cDNA was synthesized from total RNA isolated from human skin fibroblasts by reverse transcription. Partial cDNAs of human VEGF-A, VEGF-C, integrin αv, MMP3, and PAI-1 were generated by reverse transcription followed by polymerase chain reaction. First strand cDNA was synthesized from total RNA isolated from human skin fibroblasts by reverse transcription. Partial cDNAs corresponding to VEGF-A, VEGF-C, integrin αv, MMP3, and PAI-1 were generated by reverse transcription followed by polymerase chain reaction. First strand cDNA was synthesized from total RNA isolated from human skin fibroblasts by reverse transcription. Partial cDNAs of human VEGF-A, VEGF-C, integrin αv, MMP3, and PAI-1 were amplified from first strand cDNA by polymerase chain reaction using primer sets that correspond to nucleotides 198–226 and 622–590 of the human VEGF-A cDNA sequence (GenBank accession number M23977), nucleotides 1165–1191 and 1559–1533 of the human VEGF-C cDNA (GenBank accession number U43142), nucleotides 2094–2117 and 2367–2341 of the human integrin αv cDNA (GenBank accession number X08256), nucleotides 1493–1521 and 1765–1738 of the human MMP3 cDNA (GenBank accession number X05722), and nucleotides 359–381 and 1121–1098 of the human PAI-1 cDNA (GenBank accession number X04429).

cDNA Array Hybridization—The Atlas human cancer cDNA expression array kit was from CLONTECH Laboratories, Inc. (Palo Alto, CA). The array contains 588 unique human cDNAs (listed at www.CLONTECH.com/atlas/genelists/index.shtml). Human skin fibroblasts grown to near confluence were made quiescent by serum starvation, achieved by first washing the cells three times with phosphate-buffered saline to remove traces of serum factors, and then incubating the cells for 24 h in Iscove’s modified Dulbecco’s medium with no serum or protein factors added. The cells were then stimulated by adding purified recombinant Cyr61 or BSA to 10 μg/ml for an additional 24 h. Total RNA isolation, 32P-cDNA probe labeling, hybridization, and high stringency wash were all performed following protocols of the cDNA array kit provided by the manufacturer. The intensity of hybridization signals was quantified by PhosphorImager and normalized against internal controls (GAPDH and actin) on the same blot. The signal of MMP3 (positive control) normalized to GAPDH is 0.3, falling within the range of normalized signals detected for other regulated genes, which spans from 0.14 for IL-1α to 2.9 for TIMP1.

RNA Analysis, Immunoblotting, and p42/p44 MAPKs Activation—Total cellular RNA was isolated, resolved on an agarose-formaldehyde gel, and blotted onto a nylon membrane using standard protocols (18). Radioactive probes were generated by enzymatic incorporation of [32P]dCTP into indicated human cDNAs. The blots were washed at high stringency (0.2× SSPE, 0.1% SDS at 65 °C) and analyzed by PhosphorImager (Molecular Dynamics, Sunnyvale, CA). To examine secreted proteins, conditioned media were collected after cell incubation for times indicated and were centrifuged to remove cellular debris. The conditioned media were concentrated using Centricon YM-10 (molecular mass cut-off, 10 kDa), and 25 μl of the concentrate (from 0.5 ml of original conditioned medium exposed to 4 × 105 of cells) were electrophoresed on 10% SDS-polyacrylamide gel and analyzed by immunoblotting with specific antibodies using standard protocols (19).

For analysis of p42/p44 MAPKs activation, 1064SK fibroblasts were serum-starved for 24 h and treated with Cyr61 or BSA (10 μg/ml each) for 24 h. Total RNA was isolated and reverse transcribed with [32P]dATP to prepare cDNA probes, which were then hybridized to the cDNA arrays. Arrays point to examples of differential hybridization signals.

RESULTS

Identification of Cyr61-regulated Genes in Human Skin Fibroblasts Using cDNA Arrays—Primary human skin fibroblasts were grown to confluence and serum-starved prior to stimulation with either Cyr61 or BSA (10 μg/ml each) in serum-free medium for 24 h. Total RNA was isolated and used to prepare 32P-labeled cDNA probes for hybridization to cDNA arrays (Fig. 1). Negative controls of the array (blank spots, M13mp18(+) strand, ADNA, and pUC18) yielded no signal. MMP3 cDNA was represented on the array and was used as a positive control, because we have previously shown the Cyr61 up-regulates MMP3 as a cell adhesive substrate (14). cDNA array hybridization showed up-regulation of MMP3 by Cyr61,
with a fold induction consistent with results obtained by RNA blotting (see Fig. 3A). Approximately 15% of the DNA on the arrays hybridized to cDNA probes, and ~40 genes were found to be differentially expressed (>2-fold difference in signal). Of these, 12 have been confirmed by further analyses described below.

Cyr61 Up-regulates Angiogenic Factors and the Inflammatory Cytokine IL-1β—Although purified Cyr61 is pro-angiogenic in vitro and induces angiogenesis in vivo (7, 8), there has been no previous report regarding its ability to regulate the expression of angiogenic factors or cytokines. It is thus noteworthy that cDNA array analysis showed Cyr61 regulation of the potent angiogenic inducer VEGF-A, the angiogenic/lymphocytic factor VEGF-C (24, 25), and the inflammatory cytokine IL-1β. To confirm these results, human skin fibroblasts were treated with Cyr61 or BSA for 2–24 h, and gene expression was analyzed by RNA blotting. Cyr61 elevated the VEGF-A mRNA level >2-fold after 6 h and >5-fold after 24 h (Fig. 2A). VEGF-C and IL-1β mRNAs were up-regulated with slower kinetics (12–24 h), resulting in a 3–5-fold and 5–20-fold enhancement, respectively. Because VEGF-A, VEGF-C, and IL-1β are secreted proteins, their accumulation in conditioned medium was examined by immunoblotting. Significant levels of these proteins were secreted by cells treated with Cyr61 but not by BSA-treated cells (Fig. 2B). In contrast, the MMP2 (gelatinase A) level was unaffected by Cyr61 (Fig. 3). These data show, for the first time, that VEGF-A, VEGF-C, and IL-1β expression are up-regulated by Cyr61 in fibroblasts at both the mRNA and protein levels.

Expression of Extracellular Proteases and Their Inhibitors—We examined the effect of Cyr61 on fibroblast expression of secreted proteases and their naturally occurring inhibitors. RNA blotting (Fig. 3A) showed that Cyr61 treatment elevated MMP1 and MMP9 mRNA levels 3–5-fold after 12 h and >20-fold after 24 h. Up-regulation of TIMP1, an inhibitor of MMP1 and MMP3, occurred with slower kinetics and resulted in a 4-fold increase in mRNA level after 24 h. MMP2 expression, by contrast, was unaffected by Cyr61. Regulation of uPA by Cyr61 followed an unusual but consistently observed biphasic time course. The uPA mRNA level was initially unaltered (2 h) but...
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Fig. 4. Cyr61 down-regulates expression of Col1α1 and Col1α2 and up-regulates expression of integrin α3 and α5. A, skin fibroblasts were treated with Cyr61 at various concentrations from 0 to 10 μg/ml (as indicated at the top) for 24 h. Total RNA was analyzed by RNA blotting and hybridization with cDNA probes specific for human Col1α1, Col1α2, and FN. GAPDH was monitored as a loading control. B, fibroblasts were treated with Cyr61 (10 μg/ml) for various times as indicated. RNA blotting and hybridization were performed with cDNA probes specific for human integrin α3, α5, and β1 subunits. The data shown for both panels are representative of three experiments.

became suppressed by 6 h. Longer exposure (12–24 h) to Cyr61 resulted in a 3–5-fold increase in uPA mRNA. Consistent with the observation that PAI-1, a major inhibitor of uPA, is co-expressed with uPA in fibroblasts (26), Cyr61 also up-regulated PAI-1 expression by about 15–20-fold in 12–24 h (Fig. 3A). As expected, all genes tested were expressed at basal levels in control cells throughout the course of the experiments.

The accumulation of specific proteins in conditioned media of cells treated with various amounts of Cyr61 was examined by immunoblotting (Fig. 3B). Whereas the levels of MMP1, MMP3, and TIMP1 proteins were elevated by as little as 0.1 μg/ml Cyr61, up-regulation of uPA required a higher dose of Cyr61 (1 μg/ml). In each case, 5 μg/ml of Cyr61 elicited maximal accumulation of protein. The level of PAI-1 protein in conditioned medium was elevated >20-fold after 12–24 h of Cyr61 treatment, whereas the level of MMP2 protein was unaffected by Cyr61, consistent with their mRNA accumulation profiles (Fig. 3, B and C). Thus, Cyr61 treatment of human skin fibroblasts results in a robust induction of MMP1, MMP3, TIMP1, uPA, and PAI-1 in a time- and dosage-dependent manner at both the mRNA and protein levels.

Cyr61 Down-regulates Type I Collagen and Up-regulates Integrin α3 and α5 Subunits Expression—CTGF, a related protein with sequence homology to Cyr61, has been reported to mediate up-regulation of type I collagen by TGF-β (27, 28). It is thus surprising that Cyr61 down-regulates the expression of α3 and α5 subunits of human type I collagen (Col1α1 and Col1α2) in a dosage-dependent manner (Fig. 4A). The major cell surface receptors for matrix proteins such as collagen and FN are integrins, and expression of the integrin α3 and α5 subunits was found to be induced by Cyr61 via cDNA array analysis. Both integrin α3 and α5 subunits can heterodimerize with β1 subunits in fibroblasts to form receptors that bind denatured collagen and FN, respectively. Expression of integrin α5 was elevated 5-fold in 12 h and of both α3 and α5 was increased >10-fold in 24 h by Cyr61 (Fig. 4B). The level of integrin β1 mRNA, however, was unaffected by Cyr61.

Cyr61 Heparin Binding Capacity, p42/p44 MAPK Activities, and de Novo Protein and RNA Syntheses Are Required for Cyr61-regulated Gene Expression—Because the heparin binding activity of Cyr61 is indispensable for its ability to support fibroblast adhesion (9), we tested whether this activity is also necessary for Cyr61 to regulate gene expression. The recombinant protein Cyr61DM harbors mutations in the heparin-binding motifs and fails to bind heparin, is unable to support fibroblast adhesion, but is still able to mediate endothelial cell adhesion through integrin α5β3 (9) and to stimulate fibroblast migration through integrin α5β1 (10). As shown in Fig. 5A, Cyr61DM was unable to up-regulate MMP3 or integrin α5, whereas wild-type Cyr61 caused a >10-fold mRNA induction of both genes (Fig. 5A). The binding of Cyr61 to cell surface heparan sulfate may be important, because when soluble heparin was added in the culture medium to saturate the heparin-binding capacity of Cyr61, up-regulation of MMP1 and VEGF-A by Cyr61 was abrogated (Fig. 5B). These results show that the heparin binding activity of Cyr61 is indispensable for its ability to regulate the expression of at least several genes, although we cannot rule out the possibility that Cyr61 may also regulate the expression of a subset of genes through a heparin binding-independent mechanism.

Cyr61 treatment elicited a marked and sustained activation of p42/p44, evident after 12–24 h (Fig. 6A). Interestingly, the kinetics of activation are slow but consistent with the regulation of gene expression by Cyr61, which also requires 12–24 h (with the exception of VEGF-A). We therefore tested whether p42/p44 MAPKs activities are required for regulation of gene expression by Cyr61. Indeed, up-regulation of most genes by Cyr61 was severely attenuated in the presence of the extracellular signal-related kinase kinase inhibitor PD98059 (Fig. 6B). Suppression of Col1α1 mRNA, however, appeared unchanged by inhibition of extracellular signal-related kinase kinase/MAPK signaling.
The relatively slow kinetics of gene activation shown above suggested that Cyr61-mediated gene regulation is indirect and that biosynthesis of protein effectors may be required. We tested this possibility by examining the effects of cycloheximide and 5,6-dichlorobenzimidazole riboside, which inhibit translation and RNA polymerase II activity, respectively. As shown in Fig. 7, both induction of MMP1 and suppression of Col1α1 mRNA by Cyr61 were abrogated by these inhibitors. Taken together, these results show that Cyr61 regulation of gene expression requires its heparin binding activity, p42/p44 MAPK activities, de novo transcription, and synthesis of protein mediators.

Expression of Cyr61 during Cutaneous Wound Healing—The genes regulated by Cyr61 described above, including those
involved in inflammation, angiogenesis, and matrix remodeling, are known to participate in cutaneous wound healing (15–17), suggesting that Cyr61 plays a role in regulating wound repair. To investigate this possibility, we examined Cyr61 expression during cutaneous wound healing using transgenic mice that express the bacterial lacZ gene encoding β-galactosidase under the control of the endogenous Cyr61 promoter (20). The first two exons of one of the Cyr61 alleles were replaced by the lacZ gene in these heterozygous mice, which are viable and fertile and show no apparent signs of abnormality. We created full thickness incisional skin wounds on the backs of these mice, and skin samples of the wounded area were collected at various times after wounding and stained for β-galactosidase activity (Fig. 8). Little or no β-galactosidase staining was observed within the first 3 days of wounding, but strong staining was observed by the fifth day in the granulation tissue, where large numbers of fibroblasts appeared underneath the migrating and proliferating keratinocytes (Fig. 8). β-Galactosidase activity remained high 1 week after wounding, when re-epithelialization of the wound by migrating keratinocytes was complete. At this point the fibroblasts adopted myofibroblast morphology and aligned themselves in parallel bundles underneath the keratinocytes at right angles to the wound, consistent with the process of wound contraction (29). By 2 weeks after wounding, the granulation tissue was resolved, and no apparent signs of abnormality. We created full thickness incisional skin wounds on the backs of these mice, and skin samples of the wounded area were collected at various times after wounding and stained for β-galactosidase activity (Fig. 8). Little or no β-galactosidase staining was observed within the first 3 days of wounding, but strong staining was observed by the fifth day in the granulation tissue, where large numbers of fibroblasts appeared underneath the migrating and proliferating keratinocytes (Fig. 8). β-Galactosidase activity remained high 1 week after wounding, when re-epithelialization of the wound by migrating keratinocytes was complete. At this point the fibroblasts adopted myofibroblast morphology and aligned themselves in parallel bundles underneath the keratinocytes at right angles to the wound, consistent with the process of wound contraction (29). By 2 weeks after wounding, the granulation tissue was resolved, and no apparent signs of abnormality.

Cyr61 Activity in the Presence of ECM Proteins or Serum—The expression of Cyr61 in response to wounding implicates its role in cutaneous wound healing, a process that occurs over several days and in the context of ECM proteins, growth factors, and cytokines. To test whether Cyr61-regulated gene expression is modulated by the presence of ECM components, we allowed fibroblasts to adhere to dishes precoated with FN, vitronectin, laminin, or type I collagen in serum-free medium before treatment with Cyr61 or BSA for 24 h. RNA blot analysis showed that Cyr61 up-regulated MMP1 mRNA by >20-fold while suppressing Col1α1 mRNA to less than 30% of control level, irrespective of the ECM protein used as substrate (Fig. 9A). Thus, regulation of gene expression by Cyr61 was unaffected by the presence of the ECM proteins tested.

To address the effects of serum growth factors on Cyr61 activity, we added Cyr61 (5 µg/ml) to cell culture medium containing 10% fetal bovine serum. Fibroblasts were maintained in such a medium for up to 5 days, with medium renewal on day 3 as needed. Cyr61 is inducible by TGF-β1 (30), a growth factor with recognized roles in skin wound repair (15, 31). To compare the effects of TGF-β1 and Cyr61, we also added TGF-β1 (20 ng/ml) in parallel cultures. The results showed that on day 1, in contrast to serum-free cultures (Figs. 2 and 3A), Cyr61 had a minimal effect in enhancing IL-1β, VEGF-A, and MMP1 expression, whereas Col1α1 mRNA was suppressed to about 50% of control (Fig. 9B). By day 3, when cultures became nearly confluent, Cyr61 strongly enhanced IL-1β, VEGF-A, and MMP1 expression, whereas Col1α1 mRNA was suppressed to a minimal level barely detectable. These effects became even more pronounced after 5 days of culture (Fig. 9B). In contrast, TGF-β1 suppressed IL-1β and MMP1 mRNA expression to below control levels, yet slightly elevated VEGF-A, Col1α1, and MMP2 expression (Fig. 9B), and these effects of TGF-β1 were sustained throughout 5 days of culture.

Thus, even in a growth factor-rich environment (10% serum), Cyr61 can still exert its effect on up-regulate expression of IL-1β, VEGF-A, and MMP1 and suppress Col1α1 expression.

The effects of Cyr61 on gene expression lasted for at least 5 days; these kinetics closely mirror the time course of wound healing in vivo (Fig. 8). Whereas both Cyr61 and TGF-β1 enhance VEGF-A expression, major differences in their effects on gene expression were observed: 1) Cyr61 enhances but TGF-β1

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**Fig. 9.** CyR61 regulation of gene expression in different culture environments. A, skin fibroblasts were adhered to culture dishes pre-coated with 10 µg/ml each of FN, vitronectin (VN), laminin (LN), or type I collagen (Col.1) in serum-free medium for 24 h. Cyr61 or BSA was added to medium to 10 µg/ml and incubated for 24 h. Total RNA was isolated and analyzed by RNA blotting. B, proliferating fibroblasts were cultured in medium containing 10% FBS, to which Cyr61 or BSA was added to 5 µg/ml, and TGF-β1 was added to 20 ng/ml. The media were renewed on day 3 with the factors supplemented at the same level as before. At times indicated, total RNA was isolated and analyzed by RNA blotting and hybridization with specific cDNA probes as indicated. C, serum-starved fibroblasts were treated for 24 h with various concentrations (from 0 to 10 µg/ml) of Cyr61, either in the presence or absence of 20 ng/ml of TGF-β1. Total RNA was isolated and analyzed by RNA blotting. The data shown for all panels are representative of two experiments.
suppresses the expression of IL-1β and MMP1 (Fig. 9B) and 2) Cyr61 suppresses but TGF-β1 induces the expression of both Col1α1 and Col1α2 (Figs. 4 and 9B) (32, 33).

**Cyr61-regulated Gene Expression in the Presence of TGF-β1**—Although TGF-β1 can induce Cyr61 synthesis and both appear to be involved in wound repair, these factors have different effects on gene expression (Fig. 9B). Thus, we tested the effects of the combination of Cyr61 and TGF-β1 on fibroblast gene expression. Various amounts of Cyr61 (0.1–10 μg/ml) were added to serum-starved cells either alone or with TGF-β1 (20 ng/ml) for 24 h. TGF-β1 alone caused >2-fold induction of Col1α1 mRNA, whereas Cyr61 at low concentrations (0.1–1 μg/ml) had no effect (Fig. 9C). However, when Cyr61 was present at 10 μg/ml, Col1α1 expression was suppressed to 1/3 of the basal level. When both Cyr61 (10 μg/ml) and TGF-β1 were added together, Col1α1 expression became the same as basal level, which was 3-fold higher than when Cyr61 was added alone and yet 50% lower than when TGF-β1 was added alone. Thus, the opposing effects of Cyr61 and TGF-β1 neutralized one another, resulting in no change in Col1α1 expression. The expression of integrin α5 subunit was up-regulated by either TGF-β1 (–2-fold) or Cyr61 (5-fold). The presence of both Cyr61 and TGF-β1 together did not enhance integrin α5 beyond 5-fold (Fig. 9C). TGF-β1 alone suppressed MMP1 expression to below basal level, whereas Cyr61 alone (10 μg/ml) caused >15-fold elevation of MMP1 mRNA. When Cyr61 and TGF-β1 were added together, MMP1 was suppressed to an undetectable level. Thus, the effect of TGF-β1 on MMP1 expression overrode that of Cyr61. Regulation of PAI-1 expression by TGF-β1 and Cyr61 followed yet another pattern. PAI-1 expression was enhanced by either TGF-β1 or Cyr61 by a similar magnitude (–10-fold). When added together, PAI-1 expression became further elevated, and the effect of TGF-β1 and Cyr61 appeared synergistic (Fig. 9C).

These results suggest that TGF-β1 and Cyr61 induce different but interacting signaling mechanisms to regulate gene expression. Interestingly, each of the four genes tested above responded in a different manner to the mixture of Cyr61 and TGF-β1. Thus, the effects of Cyr61 and TGF-β1 on Col1α1 expression were antagonistic; in integrin α5 expression, the effects of Cyr61 and TGF-β1 overlapped; in MMP1 expression, TGF-β1 completely suppressed the strong inducing effect of Cyr61; and in PAI-1 expression, effects of Cyr61 and TGF-β1 were synergistic. These results indicate a complex interaction between the signaling pathways induced by Cyr61 and TGF-β1.

**DISCUSSION**

The principal finding of this study is that Cyr61, an ECM-associated angiogenic protein, regulates the expression of a genetic program for wound healing in fibroblasts. This conclusion is based on two observations: 1) the Cyr61 gene is inducibly expressed in granulation tissue during wound repair and 2) Cyr61 regulates the expression of genes involved in angiogenesis, inflammation, matrix remodeling, and cell-matrix interactions. Thus, the roles of Cyr61 in wound healing extend beyond its activity as an angiogenic inducer and may coordinate multiple events through regulation of specific genes.

Purified Cyr61 elicits gene expression changes in human fibroblasts in serum-free medium in a dose-dependent manner. Site-specific mutations in the Cyr61 polypeptide abolished its ability to regulate gene expression, demonstrating that this activity is an intrinsic property of the Cyr61 polypeptide (Fig. 5A). Cyr61 most likely acts as a matricellular protein (34) rather than a conventional growth factor or cytokine, given that it: 1) associates with the ECM upon secretion (35), 2) is structurally related to other ECM proteins such as von Willebrand factor and thrombospondin, 3) supports cell adhesion and induces adhesive signaling, and 4) acts through binding to integrin receptors. The dosage requirements for Cyr61 to regulate different genes vary, ranging from 0.1 to 1 μg/ml (–2.5–25 nm) for vPA and MMPs (Fig. 3B) to 1–5 μg/ml for IL-1β, Col1α1, and Col1α2 (Fig. 4A and data not shown). These effective concentrations are higher than those required of soluble growth factors and cytokines but consistent with the dosage requirements for other matricellular and ECM proteins, such as tenascin, SPARC, collagen, laminin, and the 120-kDa FN fragment, to regulate gene expression (36–43).

Although Cyr61 expression is strongly induced by mitogenic signals in fibroblasts (1), it should be noted that the actual local concentration of Cyr61 as an ECM-associated molecule in tissues has been difficult to assess. Cyr61 binds heparin with high affinity (35), and the interaction of Cyr61 with cell surface HSPGs is required for it to support fibroblast adhesion (9). Two lines of evidence showed that Cyr61 regulation of gene expression is also dependent on its capacity to bind heparin: 1) a heparin-binding defective mutant of Cyr61 was unable to regulate gene expression (Fig. 5A) and 2) soluble heparin, in quantities sufficient to saturate the heparin-binding site of Cyr61, abrogated its ability to enhance gene expression (Fig. 5B). Interaction with HSPGs alone may not be sufficient, however, because numerous extracellular proteins also can bind heparin but do not have the same activities. It is likely that another cell surface receptor(s), together with HSPGs, may be necessary to determine the specific effects of Cyr61 on gene expression. It is of interest to note that cell surface HSPGs are important for wound healing. For instance, null mutation of the syndecan-4 gene results in dramatically delayed skin wound healing in mice (22).

Although p42/p44 MAPKs were activated by Cyr61 (Fig. 6A), the unusually slow kinetics (more than 6 h of exposure to Cyr61 before activation occurs) suggest that activation is mediated through secondary factor(s) induced by Cyr61. This is consistent with the observation that the gene regulation activity of Cyr61 was abrogated by inhibitors of transcription or protein synthesis (Fig. 7). Because the extracellular signal-related kinase inhibitor, PD98059, severely blunted Cyr61 enhancement of gene expression (Fig. 6B), it follows that the activities of p42/p44 MAPKs may be required for Cyr61 to regulate gene expression. Functional AP-1 elements have been found in the promoter regions of genes encoding IL-1α, VEGF-A, and MMP1, and p42/p44 MAPKs were shown to activate transcription through these AP-1 elements (44–46). Although p42/p44 MAPKs are also involved in the down-regulation of type 1 collagen gene expression by basic fibroblast growth factor, platelet-derived growth factor, and ceramide (47, 48), PD98059 was unable to block Cyr61-suppression of Col1α1 expression (Fig. 6B). Therefore, alternative pathways for the down-regulation of type 1 collagen must exist. Interestingly, CTGF, a protein closely related to Cyr61 and also expressed in fibroblasts (1), it should be noted that the actual local concentration of Cyr61 as an ECM-associated molecule in tissues has been difficult to assess.
TGF-β1 are released by activated platelets and damaged tissue upon wounding, the actions of these growth factors may explain the expression of Cyr61 and CTGF in wounds (Fig. 8) (54, 55). The kinetics of Cyr61-regulated gene expression are consistent with the wound healing process in vivo. Among the first genes up-regulated by Cyr61 is the angiogenic factor VEGF-A (6 h), consistent with the need for nutrient and oxygen supply to cells of the healing wound. Subsequent expression of VEGF-C may enhance the angiogenic process and/or promote lymph vessel formation (24, 25). Although Cyr61 up-regulates both MMPs and their natural inhibitor TIMP1, expression of the proteases (MMP1 and MMP3) precedes that of the inhibitor (TIMP1) (Fig. 3A). We speculate that elevation of proteases prior to their inhibitors allows ECM degradation to occur in a temporally regulated fashion.

A number of ECM proteins are capable of regulating MMP expression in fibroblasts, endothelial cells, or macrophages; these include type 1 collagen (56, 57), FN proteolytic fragments (37, 38, 58), laminin (39), SPARC (36), tenascin-C (59), and thrombospondin-2 (60). It is of interest to note that FN fragments are capable of up-regulating fibroblast expression of MMPs, IL-1α, and IL-1β but do not affect expression of angiogenic factors such as VEGF-A (38, 58). To our knowledge, Cyr61 is the only matricellular protein (34) capable of regulating genes that play roles encompassing several major processes in wound repair, including angiogenesis, inflammation, tissue remodeling, and cell-ECM interaction. Furthermore, only Cyr61 has been shown to have long term effects on gene regulation lasting for at least 5 days (Fig. 9B), consistent with the time course of wound healing in vivo. Importantly, the effects of Cyr61 on gene expression were observed even in cells grown in 10% serum (Fig. 9B), showing that Cyr61 actions are not mitigated by the presence of serum growth factors. Thus, Cyr61 appears to have more diverse functions in skin wound healing compared with other ECM-associated proteins examined to date.

Even though Cyr61 is an immediate-early gene inducible by TGF-β1, the effects of Cyr61 and TGF-β1 on fibroblast gene expression are not identical. Where both are present, the effects of Cyr61 and TGF-β1 on fibroblast gene expression can be antagonistic, additive, or synergistic. The effect of TGF-β1 on MMP1 expression completely overrides that of Cyr61 (Fig. 9C). During skin wound healing, the peak of TGF-β1 expression correlates with granulation tissue formation when ECM proteins are being actively produced and deposited, whereas the peak of Cyr61 expression occurs about 3 days later, coincident with ECM remodeling and granulation tissue contraction (Fig. 8) (31). We speculate that fibroblasts produce more collagen and fewer MMPs during the early phase of wound healing when the level of TGF-β1 is high and that of Cyr61 low, thus favoring a net accumulation of ECM and granulation tissue formation. Subsequently, when TGF-β1 level decreases and Cyr61 is highly expressed, collagen expression is decreased, whereas MMP expression is increased, thus facilitating ECM remodeling.

Based on the current study and information available in literature, we propose a working model for the mechanism of Cyr61 action in cutaneous wound healing (Fig. 10). After injury, Cyr61 is produced both by dermal fibroblasts (Fig. 8) and endothelial cells.2 As an ECM-associated signaling protein (8, 35), Cyr61 interacts with integrin receptors to induce adhesive signaling (9, 14), cell migration, and enhancement of growth factor-induced mitogenesis (10). Although Cyr61 interacts directly with endothelial cells through integrin α5β3 to induce pro-angiogenic activities (7, 8), it also up-regulates synthesis of VEGF-A and VEGF-C in fibroblasts (Fig. 2), further enhancing the angiogenic potential of the immediate milieu. In addition, Cyr61 enhances fibroblast synthesis of IL-1β, which may act upon macrophages to enhance the inflammatory response. It is of interest to note that macrophage is an important source of growth factors necessary to stimulate fibroplasia and angiogenesis in granulation tissue (16). In addition, exposure of fibroblasts to Cyr61 leads to elevated expression of ECM-degrading proteases and their inhibitors, including MMP1, MMP3, TIMP1, uPA, and PAI-1, and down-regulation of type 1 collagen (Figs. 3 and 4A), thereby leading to ECM remodeling. The expression of provisional matrix integrin subunits α5 and α6 is enhanced by Cyr61 as well (Fig. 4B). This hypothetical model proposes that in response to wounding, Cyr61 is induced and subsequently activates genes that play multiple and coordinated roles in wound healing, including angiogenesis, inflammation, and ECM remodeling (Fig. 10). The functional roles of Cyr61 in cutaneous wound healing clearly merit further investigation in the context of wounding models in whole animals.

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