Tendon Healing *In Vivo*: Gene Expression and Production of Multiple Growth Factors in Early Tendon Healing Period

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**Purpose** The actions of growth factors during healing of injured flexor tendons are not well characterized, although information pertinent to some individual growth factors is available. We studied gene expression and protein production of a number of growth factors at several time points during the early healing period in a chicken model.

**Methods** Seventy-four long toes of 37 white Leghorn chickens were used. The flexor digitorum profundus tendons of 60 toes were surgically repaired after complete transection and were harvested for analysis 3, 5, 7, 9, 14, and 21 days after surgery. The expression of 6 growth factors was studied at 4 time points after surgery with real-time quantitative polymerase chain reactions, and production and distribution of 3 growth factors at all 6 time points were studied by immunohistochemical staining with antibodies. Fourteen tendons that had no surgery served as day 0 controls. Tendon healing status was also assessed histologically.

**Results** Throughout the early tendon healing period, connective tissue growth factor (CTGF) and transforming growth factor β (TGF-β) showed high levels of gene expression. Levels of gene expression of vascular endothelial growth factor (VEGF) and insulin-like growth factor 1 (IGF-1) were high or moderately high. Expression of the TGF-β gene was upregulated after injury, whereas the basic fibroblast growth factor (bFGF) gene was downregulated at all postsurgical time points and expressed at the lowest levels among 6 growth factor genes 2 to 3 weeks after surgery. The platelet-derived growth factor B (PDGF-B) gene was also minimally expressed. Findings of immunohistochemistry corresponded to TGF-β, bFGF, and IGF-1 gene expression.

**Conclusions** In this model, up to 3 weeks after surgery, gene expression and production of TGF-β are high and are upregulated in this healing period. However, expression of the bFGF gene and protein is low and decreases in the healing tendon. The CTGF, VEGF, and IGF-1 genes are expressed at high or moderately high levels, but PDGF-B is minimally expressed. *(J Hand Surg 2008;33A:1834–1842. Copyright © 2008 by the American Society for Surgery of the Hand. All rights reserved.)*

**Key words** Flexor tendon, gene expression, growth factors, early healing period.
The potential of growth factor-related therapies to augment the early tendon healing process has attracted the attention of hand surgeons.1–7 Flexor tendons, the intrasynovial tendon segments in particular, have limited vascular supply and cellularity and generally have low growth-factor activity. Ruptures and adhesions of the repaired tendon baffling surgeons are attributed to poor intrinsic healing capacity, which precludes efficient and risk-free tendon mobilization early after surgery.8–11 Promotion of tendon healing through molecular approaches and seeking potential targets of molecular modulation should be based on a thorough understanding of the activities of all factors involved. Nevertheless, our information about molecules potentially serving as targets of efficient regulation is limited. In terms of activities of growth factors involved in tendon healing, we have information only on changes in individual growth factor genes from separate sets of studies or expression of several growth factors at a single time point.12–17

Chang et al. studied protein levels of TGF-β1 from 1 to 56 days after surgery in injured rabbit flexor tendons and sheaths. They found upregulation of this factor in both intrinsic tenocytes and extrinsic fibroblasts in the sheaths. Duffy et al. detected bFGF and PDGF in canine digital flexor tendons 3, 10, and 17 days after tendon repairs. Subsequently, Chang et al. further characterized the presence of bFGF in the injured rabbit digital flexor tendon and found upregulation of bFGF in the tendon wound. Bidder et al. and Boyer et al., respectively, investigated gene expression of vascular endothelial growth factor (VEGF) at and around the tenorrhaphy site of flexor tendon repair in a canine model. Using immunohistochemical staining, Tsubone et al. studied the levels of a number of growth factors such as TGF-β, epidermal growth factor, and IGF in flexor tendons at a single time point—10 days after surgery.

The earlier investigations addressed the changes of growth factors only at a single time point or the changes of a single growth factor in the healing period. In addition, quantitative analysis of gene expression has not been included, because precise measurement of gene expression by a quantitative method was not popularly available at that time. There has been a lack of investigation of the relative levels of expression of these growth factors through the early healing period. Our knowledge regarding general pictures of differences in gene expression and the levels of growth factors throughout the early healing period is scarce. In this study, we studied gene expression and protein productions of a number of growth factors at several time points during the early period of tendon healing in a chicken model. We hypothesized that the relative levels of gene expression and productions of growth factor genes vary considerably during the early tendon healing process. Six growth factors—bFGF, TGF-β, PDGF, VEGF, IGF-1, and CTGF—were studied because their function has attracted popular attention in studying digital flexor tendon healing.12–17 Roles of other growth factors such as bone morphogenetic protein-14, -12, and -2 and cartilage-derived morphogenetic protein 218–21 have been studied in tendon healing in other parts of the body or tendon–bone junction; these growth factors were not included. The study may help us understand how growth factors orchestrate the tendon healing process and identify potential targets of molecular regulation of tendon healing.

**MATERIALS AND METHODS**

Seventy-four long toes of both feet of 37 white Leghorn chickens were used. The chickens, weighing 1.5 to 2.5 kg each, were used as the experimental model; they have a flexor mechanism similar to that of human digits.22,23 The chickens were anesthetized with intramuscular injection of ketamine (50 mg/kg body weight). Institutional animal research regulations were followed during the study.

**Tendon injury, surgical repairs, and sample harvesting**

Surgery on the long toes of both feet of 30 chickens was performed using an aseptic surgical technique. A tourniquet, using an elastic bandage, was applied over the thigh during surgery. A zigzag incision was made in the plantar skin between the proximal interphalangeal and distal interphalangeal joint levels of the toes. This area corresponds to zone II of the human hand. Through a 1-cm longitudinal incision in the sheath, the flexor digitorum profundus tendon was transected completely with a fine blade and was surgically repaired with 5–0 sutures (Ethilon, Ethicon, Somerville, NJ) by a modified Kessler method. Peripheral sutures were added to the tendons with 6–0 sutures, and the sheath was not repaired. After the skin incision was closed with sutures, the toes were immobilized in semiflexion by cast.

At 3, 5, 7, 9, 14, and 21 days after surgery, a total of 60 tendon samples were harvested. No repairs were found ruptured at the time of harvesting. Fourteen tendons (7 chickens) having no surgery were harvested as day 0 controls. A pilot study showed that sample sizes of 6 or greater are needed to ensure a power over 0.80 for comparisons of gene expression. The samples for this analysis, therefore, were 6 to 8 at each time point. The number of tendons harvested is detailed in Table 1.
A total of 32 tendons were harvested for analysis of gene expression, and the other 42 tendons from 21 chickens were harvested for immunohistological staining and conventional histology. During harvest, the tendons were exposed through an incision in the skin. A 2-cm tendon segment with the transaction site at its center part was harvested from each long toe. When adhesions presented over the surface of repaired tendons for analysis of gene expression, the adhesions were removed. Day 0 control tendons were taken from the area identical to those from the surgical toes. The samples were stored in liquid nitrogen for analysis of gene expression or were fixed in 4% paraformaldehyde at 4°C for immunohistochemistry.

**Analysis of expression of multiple growth factor genes**

Tendon samples were thawed, dissected, and homogenized on ice. Total RNA was isolated with Trizol (Life Technologies Ltd, Paisley, UK) and was transcripted to complementary DNA using a ThermoScript RT-PCR system (Invitrogen, Carlsbad, CA). Expression of growth factor genes was analyzed by real-time quantitative polymerase chain reactions (qPCR) using the RotorGene 3000 (Corbett Research Pty Ltd, Sydney, Australia). EvaGreen (Biotium Inc, Hayward, CA) served as a dye that binds to amplified DNA to emit fluorescence during reactions. EvaGreen recently emerged as an optimal green fluorescent DNA dye for qPCR; it has equal or better sensitivity compared with SYBR Green I. The reaction mixture of 25 μL contained 12.5 μL EvaGreen qPCR Master Mix (Biotium Inc, Hayward, CA), 1 μL primers (10 μM), 1 μL template complementary DNA, and 10.5 μL doubly distilled water. The glyceraldehyde-3′ phosphate dehydrogenase (GAPDH) gene served as an internal control for expression levels of target growth factor genes. Primers were synthesized to amplify a gene segment of GAPDH of 139 base pairs (bp): 5′-aagctgagaacgggaaactac-3′ (left) and 5′-ccagctgacaagc-3′ (right). The primers were designed based on sequences from the Genebank database. The lengths of amplified segments and primers for growth factor genes are as follows: left primers were listed first, followed by right primers. A 143-bp segment of the TGF-β gene was amplified with 5′-gtacctgactatctgtggaagct-3′ and 5′-gcttattggag-3′. A 97-bp segment of CTGF gene was amplified with 5′-gtctggtccaaacccctgt-3′ and 5′-acattgcagtctcgctcg-3′. A 172-bp segment of the VEGF gene was amplified with 5′-caagttcagtgcttgtaagc-3′ and 5′-gtgcttgctccaagacctgt-3′. A 132-bp segment of the PDGF-B gene was amplified with 5′-gagcccataccggaatt-3′ and 5′-tcgattcgcttgctga-3′. An 88-bp segment of the IGF-1 gene was amplified with 5′-caacggtgcctgggtggc-3′ and 5′-gattagttggaacct-3′. A 190-bp segment of the bFGF gene was amplified with 5′-cacaacctctgtgcaagctg-3′ and 5′-agactgttgacacacc-3′.

After an initial incubation for 15 minutes at 95°C, the reactions were carried out for 40 cycles at 95°C for 15 seconds and 60°C for 45 seconds (fluorescence collection). We set the threshold at the level at the middle steady portion of reaction cycles–versus–fluorescence curve, and we calculated the cycle threshold values of target genes using customized software (Rotor Gene Analysis Software 6.0, Corbett Research Pty Ltd.). To account for variability in total RNA input, the expression of the transcriptions were normalized to the GAPDH gene to standardize comparison. Finally, the PCR products were separated on 1.5% agarose gel electrophoresis in the presence of ethidium bromide, visualized on an ultraviolet illuminator to verify product sizes, and recorded.

**Immunohistochemical staining**

Tendons fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) were placed on ice for 2 hours, followed by saturation in 20% sucrose at 4°C. The samples were embedded in paraffin and cut longitudinally to 4 μm. The sections were washed with PBS and then subjected to staining with different primary antibodies. The sections were stained for IGF-1 using monoclonal mouse anti-chicken antibodies (Upstate Biotechnology, Lake Placid, NY) at a concentration of 10 μg/ml. The sections were stained for bFGF using polyclonal mouse anti-chicken antibodies (BD Biosciences, San Jose, CA) at a dilution of 1:100. The

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**TABLE 1. Sample Sizes for Gene and Protein Expression at Each Evaluation Time Point**

<table>
<thead>
<tr>
<th>Evaluation</th>
<th>Day 0*</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
<th>Day 9</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Total</th>
</tr>
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<tr>
<td>Gene expression</td>
<td>8</td>
<td>6</td>
<td>—</td>
<td>—</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>32</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>42</td>
</tr>
</tbody>
</table>

*Day 0 samples are normal tendons.
sections were stained for TGF-β using polyclonal goat anti-chicken antibodies (Neuromics, Bloomington, MN) at a concentration of 15 μg/ml. The primary antibodies were applied and incubated for 30 minutes at room temperature. The sections were washed again with PBS and incubated in the biotinylated secondary antibodies for 30 minutes from 2 kits: an anti-goat ABC staining system (sc-2023, Santa Cruz Biotechnology Inc, Santa Cruz, CA) for TGF-β and an anti-mouse ABC staining system (sc-2017, Santa Cruz Biotechnology, Inc) for bFGF and IGF-1. After 3 washes with PBS, the sections were incubated in enzyme reagents, which combine 50 μL avidin, 50 μL biotinylated HRP, and 2.5 mL PBS. Subsequently, they were incubated in a few drops of peroxidase substrate mixing liquid and were washed in deionized water. Finally, sections were counterstained in hematoxylin followed by several washes with deionized water. In negative controls, nonimmune serum was used instead of primary antibodies.

The sections of the samples at different time points were also stained with hematoxylin-eosin. Tendon morphology based on conventional histology was also studied to assess the healing status of the tendons and inflammatory changes from day 0 to day 21. Inclusion of these sections was to observe essential features of tissue reactions in the 3-week period, particularly the length of inflammatory changes and timing of connection of collagen across the tendon repair site, to provide some background information pertinent to tendon healing in this model.

The histological sections were observed under a microscope (Leica DMR 3000; Leica Microsystem, Bensheim, Germany). The general density of immunohistological staining, location, and distribution of the stained growth factors in epitenon and endotenon were observed. Differences in the amount of 3 growth factors were compared at all 7 evaluation time points.

**DATA ANALYSIS**

Levels of expression of target growth factor genes were recorded as relative quantitation of target gene expression, normalized by the endogenous reference (GAPDH gene). The data were expressed as means and standard deviations, and the significance level was set at p < .05. Two-way analysis of variance was used to compare differences among the data. A Tukey test was used as a post hoc test to detect significance between each pair of data from different time points of a given growth factor gene. A paired t-test was used to compare pairs of data of growth factor genes at an identical evaluation time point. Statistical analyses were conducted with a Stata 7.0 software package (Stata Corp, College Station, TX).

**RESULTS**

**Different levels of expression of growth factor genes in the early healing period**

Throughout the early healing period, CTGF showed the highest levels of gene expression among 6 studied growth factors (p < .05 or p < .01 for all comparisons) (Fig. 1), except that the levels of expression of the TGF-β gene were statistically the same as those of CTGF at day 3. In the normal tendon, CTGF did show statistically higher levels of gene expression than TGF-β (p < .01). In the healing tendons, expression levels of the CTGF and TGF-β genes were 10 to 100 times those of the bFGF and PDGF-B genes, and the differences were statistically significant at all postsurgical time points (p < .01 or p < .001). During the early healing period, levels of expression of the VEGF and IGF-1 genes were similar and were significantly higher than those of bFGF and PDGF-B at all 4 postoperative time points (p < .01 or p < .001).

Levels of expression of the PDGF-B and bFGF genes were generally low during the healing process (Fig. 1). At day 0, expression of the bFGF gene was the second lowest; its expression levels were higher than those of the PDGF-B gene, but it declined drastically as tendon healing proceeded. Levels of expression of the bFGF gene from day 3 to day 21 were only about 1/5 to 1/20 of that at day 0. Changes in the levels of expression of the bFGF gene were statistically significant both from days 0 to 3 and from days 3 to 9 (p < .05, both comparisons). The bFGF gene expressed at the lowest levels at postsurgical days 14 and 21 among all growth factors (p < .01).

Throughout this healing stage, expression of the PDGF-B gene was at minimal levels. Expression of the PDGF-B gene was the lowest at day 0, which was even lower than that of the bFGF gene. However, as the healing initiated, expression levels of the PDGF-B gene increased progressively, although its overall expression remained at low levels (Fig. 1).

These 6 growth factor genes also exhibited different responses to tendon injury and different regulatory changes during the healing period. Expression of the TGF-β gene was upregulated from day 3 to day 21 compared with its expression levels at day 0. In contrast, the bFGF gene was downregulated at all postsurgical time points. In this study, day 0 samples had no injury and no healing occurred; gene expression profiles.
of day 0 samples served as baselines to determine up- or downregulation during the healing process.

Changes in gene expression of each growth factor over the 3-week period

We observed some distinct patterns of changes in expression of these growth factor genes at different time points over the 3-week period (Fig. 2). Except for the bFGF gene, expression of other growth factor genes was upregulated at day 3 (p < .05 or p < .01). Their levels of gene expression at day 3 were 2 to 50 times those at day 0, with the smallest increases seen in the CTGF gene and the greatest increases seen in the IGF-1 gene.

After 3 days, expression of all genes that initially showed increased expression at day 3 declined (Figs. 1 and 2). Levels of expression of CTGF and VEGF genes were only about 1/3 to 1/10 of those at day 0 respectively, but the PDGF-B and IGF-1 genes showed expression levels higher at days 9 to 21 than at day 0.

Unlike other growth factors, the bFGF gene showed no increases during the healing process, even at day 3. Unexpectedly, it showed a continuous and sharp decrease in the levels of gene expression throughout this healing period (Fig. 2). On days 3 to 21, levels of expression of bFGF gene ranged from 1/3 to 1/200 that of day 0. Obviously, the bFGF gene exhibited a unique expression pattern, progressively declining as the healing progressed.

Immunohistochemistry

Regarding immunohistochemical findings corresponding to TGF-β, bFGF, and IGF-1 gene expression, we noted increased intensity of positive staining by TGF-β antibodies from day 3 to day 21 over that at day 0, but there was no increase in intensity of stained bFGF in samples from day 3 to day 21 compared with those at day 0 (Fig. 3). Among the sections stained with TGF-β, bFGF, and IGF-1 antibodies, intensity of positive staining was the highest in the sections stained with TGF-β antibodies, and the sections stained with bFGF antibodies showed the weakest staining (Fig. 3). The samples stained with IGF-1 antibodies showed weak staining at day 0 and moderately dense staining at days 3 to 7, and the intensity of positive staining decreased at days 14 and 21 (Fig. 3). The changes in staining in epitendon of the samples were rather consistent with those in the endotenon (Fig. 4).

Tendon healing status and inflammatory changes

In sections stained with hematoxylin-eosin, we observed that inflammatory changes peaked at day 3 and subsided drastically after day 9. Intratendinous collagen bundles aligned better at day 21 than at days 3 to 14. From day 9 to day 14, collagen fibers began to bridge the tendon repair sites. Samples at day 21 showed distinct signs of tendon healing in terms of solid connection of collagens across the repair sites.
DISCUSSION

In this study, we found distinctly different patterns of gene expression among the 6 growth factors during the early tendon healing process (Table 2). Patterns of expression of TGF-β, CTGF, VEGF, and IGF-1 genes are clearly different from those of PDGF-B and bFGF genes. Levels of expression of TGF-β, CTGF, VEGF, and IGF-1 genes were comparatively high in uninjured tendons, with upregulation of these genes after injury, in contrast to minimal expression of PDGF-B and bFGF genes in uninjured tendons and downregulation of already low levels of expression of the bFGF gene after tendon injury. Previous investigators have noted distinct increases in the amount of mRNA in a rabbit tendon injury model using in situ hybridization. Findings of postsurgical upregulation of TGF-β gene expression in our model are consistent with this previous observation. The TGF-β gene has been considered to be associated with tissue fibrosis, related to scar and adhesions around tendons.
The CTGF gene is a factor rarely studied in association with the early flexor tendon healing process. Expression of the CTGF gene in lacerated digital flexor tendons has not been reported, although changes in its expression were noted in tendons subjected to cyclic loading.24–25 Here we found that among 6 studied growth factors, levels of expression of the CTGF gene actually are the highest in uninjured tendons. This probably indicates critical roles of this growth factor in maintaining basic biological processes of connective tissues such as the tendon. Levels of expression of this growth factor gene were about 1/10 of those of GAPDH, a housekeeping gene, and were higher than those of TGF-β. The roles of CTGF in many other tissues have been characterized.26–28 The CTGF gene likely is essential to basic biological activities in tendons, as well. Some investigators believe CTGF to play a role in extracellular matrix production, leading to fibrotic disorders of many types of tissues.28–31 Whether this growth factor plays a critical role in formation of adhesions is not yet known.

The bFGF gene is expressed minimally in the uninjured tendon as well as during the early healing period, and it is further downregulated after tendon injury. The expression pattern of bFGF is contrary to what is seen in repair processes of many other tissues, in which expression of growth factors increases after injury.32–35 This represents a unique finding in expression profiles associated with the intrasynovial digital flexor tendon healing process. It is well documented that intrasynovial tendon segments have poor innate healing capacity, leading to weakness in biological repair strength during the early healing period. Although definite characterization of the roles of bFGF in relation to tendon healing strength may require silencing other growth factors, it is reasonable to speculate that low bFGF activity is—at least partly—responsible for low strength during the early tendon healing period.

Among 6 growth factors studied in uninjured normal tendons, levels of expression of the PDGF-B gene were the lowest and were persistently low during the tendon healing process, although levels were slightly upregulated after injury. The roles of PDGF-B in tendon healing processes remain poorly understood. It is possible that low levels of PDGF-B may relate to the insufficient healing capacity of the injured tendon. Two recent studies in canine models showed that addition of exogenous PDGF-B to injured intrasynovial flexor tendon enhances tenocyte proliferation and collagen remodeling.36,37 The VEGF and IGF-1 genes showed
changes in gene expression profiles similar to those of TGF-β; VEGF and IGF-1 were activated during tendon repair processes. It is clear that even activation of these growth factor genes (TGF-β, VEGF, CTGF, and IGF-1) likely fails to increase healing strength efficiently during the early tendon healing period. Activation of some of them may be detrimental, leading to adhesion formations.

Focusing on the potential contribution of any single growth factor to either formation of adhesions or weak healing strength may oversimplify the complex interactions of these growth factors. It is likely that these factors work in groups, each playing an integral role in the tendon healing process. In a previous report, transfer of exogenous VEGF gene to proliferating tenocytes increased expression of the endogenous TGF-β gene.\(^3\) This finding highlights the possibility that a group of genes such as TGF-β and VEGF (both potent stimulators of collagen deposition and angiogenesis) work synergistically. Previously, to reduce adhesion formation, investigators tried to neutralize the activities of TGF-β.\(^3\) In light of the current findings, it might also be reasonable to silence the effects of 1 or a group of other growth factors—VEGF, CTGF, or IGF-1. Targeting regulation of activities of 1 growth factor has been the mainstay of past efforts.\(^2,4,38–40\) Silencing the activities of more than 1 growth factor is likely to prove an even more effective approach.

Augmentation of the capability of tenocytes to produce a tendon matrix was attempted in vitro by means of gene transfers.\(^2,38,39\) Transfer of either exogenous bFGF or PDGF-B genes considerably enhanced production of type I and type III collagens,\(^2,38\) but transfer of the VEGF gene did not promote production of these collagens.\(^39\) Taken together, the present findings and those from previously reported studies suggest that the tendon may not be short of VEGF during the early healing period. Addition of this factor is not critical to the healing strength. However, when intrinsic bFGF and PDGF-B are insufficient, supplementation of 1 or both growth factors favors collagen production and stronger tendon healing. It is quite likely that supernormal amounts of bFGF and PDGF-B, produced persistently within the healing tendon by transfer of exogenous bFGF and PDGF-B genes, would ameliorate innate shortages of these growth factors during the early tendon healing process. Our present study provides some information pertinent to these 6 growth factors, based on which further molecular modulation of tendon healing can be planned.

### REFERENCES


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**TABLE 2. Expression of Growth Factor Genes**

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>Day 0</th>
<th>Day 3</th>
<th>Day 9</th>
<th>Day 14</th>
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<td>Highest</td>
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<td>High</td>
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<tr>
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<td>Moderate</td>
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</tr>
<tr>
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</tr>
<tr>
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<tr>
<td>PDGF-B</td>
<td>Lowest</td>
<td>Lowest</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
</tr>
</tbody>
</table>

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27. Brigstock DR. Regulation of angiogenesis and endothelial cell function by connective tissue growth factor (CTGF) and cysteine-rich 61 (CYR61). Angiogenesis 2002;5:153–165.