

# Abstracts

## Abstracts from the Second International Workshop on the CCN Family of Genes

### 01 RHOA-DEPENDENT REGULATION OF CYR61 GENE EXPRESSION INVOLVES AP-1- AND CREB-DEPENDENT PROMOTER ACTIVATION IN SMOOTH MUSCLE CELLS

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We have previously demonstrated that mechanical strain strongly upregulates the expression of the Cyr61 gene in cultured smooth muscle cells and suggested a potential key role of the small G-protein RhoA in strain-induced Cyr61 gene expression. Since the activation of the RhoA signaling pathway is a critical mechanism for regulating smooth muscle cell differentiation, expression of the Cyr61 gene is probably involved in the progression of these cells to hypertrophy. In this study, we sought to investigate further the mechanisms whereby RhoA activation regulates Cyr61 gene expression and the signaling machinery involved in such a process.

First, we showed that constitutive expression of an active form of RhoA increased endogenous levels of Cyr61 mRNA in a dose-dependent manner. Similarly, incubation of the cells with exogenous inducers of Rho activation such as sphingosine 1-phosphate (S1P) or lysophosphatidic acid (LPA) upregulate the expression of the Cyr61 gene at both the mRNA and protein levels. Y-27632, a well known RhoA inhibitor, abolished their effects. When actinomycin D was added to the cells concomitantly with either S1P or LPA, the decay in Cyr61 transcripts was not altered, indicating the lack of independent effects on transcript stability. Nuclear run-on analysis further indicated that RhoA-dependent Cyr61 gene expression occurs at the level of transcription.

We further studied the transcriptional requirements for RhoA-induced Cyr61 gene expression by using a construct containing a 936-bp DNA fragment of the promoter region of the human Cyr61 gene cloned upstream of a CAT reporter. The relative CAT activities driven by this promoter region were measured in transiently transfected cells. We showed that coexpression of constitutively active RhoA but not Rac 1 or cdc 42, significantly increased (11- to 15-fold) the activity of the Cyr61 promoter. The latter was enhanced when the cells were treated with either S1P or LPA as well. To define the cis-acting sequences involved in this promoter activation, point mutations of several putative regulatory sequences were introduced in the context of the Cyr61 promoter-reporter construct.

We found that RhoA-dependent activation of the Cyr61 promoter was reduced when either a putative proximal CREB/JUN sequence or a distal AP-1 site was mutated, and completely abolished when both the CREB/JUN and AP-1 sequences were mutated. Coexpression of a dominant negative mutant of c-fos decreased the RhoA-induced wild type promoter activity. On the other hand, constitutive expression of an active form of CREB enhanced the promoter activity.

Additionally, treatment of the cells with anisomycin, a potent activator of AP-1 and CREB, strongly upregulated the expression of either the endogenous Cyr61 gene or the transfected Cyr61 promoter-reporter construct. Furthermore, with the use of pharmacological inhibitors that interfere with known signaling pathways (SP600125, SB203580) we showed that transduction signals through Jun N-terminal kinase (JNK) and p38 are potentially involved in Cyr61 gene activation through RhoA. Co-expression of an active form of either MKK3 or MKK6, upstream activators of p38, increased the Cyr61 promoter activity. Taken together, these results provide evidence that in smooth muscle cells RhoA-dependent regulation of Cyr61 gene involves signaling through JNK and p38, as well as the AP-1 and CREB transcription factors as downstream effectors.

### 02 PARACRINE REGULATION OF CTGF IN THE RAT OVARY

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The ovary is a dynamic structure, with waves of follicular development and repeated ovulations, which necessitate constant tissue

remodelling and make this organ a likely candidate for CTGF involvement in repeated injury/repair cycles. Using differential display reverse transcription polymerase chain reaction, we have recently identified CTGF as an expressed gene in rat ovarian granulosa cells.<sup>1</sup> However nothing is known of the regulation of CTGF in the ovary.

We looked at the effects of endocrine (follicle-stimulating hormone (FSH)), paracrine (transforming growth factor  $\beta$ 1 (TGF $\beta$ -1) and androgen (dihydrotestosterone; DHT) of theca cell origin, growth differentiation factor 9 (GDF-9) of oocyte origin) and autocrine (activin-A of granulosa cell origin) factors on CTGF expression and P450 aromatase activity (an index of cellular differentiation) in granulosa cells isolated from DES-treated (stimulates preantral/early antral follicular development) 21 day Wistar rats, cultured for 6–48 hours in vitro.

After 48 hours, TGF $\beta$ -1 (0.1–10.0 ng/ml) stimulated CTGF mRNA expression in a dose-dependent manner by up to 10-fold, without altering aromatase activity. FSH (10 ng/ml) and dibutyryl cAMP (dbcAMP) (1 mM) suppressed expression by 20–30%, whereas DHT (1  $\mu$ M) stimulated expression by 50%. DHT combined with FSH or dbcAMP further suppressed expression. Aromatase activity was increased threefold by FSH alone, but 20-fold by FSH plus DHT. FSH (0.1–10.0 ng/ml) dose-dependently inhibited the stimulatory action of TGF $\beta$ -1, reducing CTGF mRNA by up to 90%, while stimulating aromatase activity up to 100-fold. Individual actions of FSH and TGF $\beta$ -1 on CTGF mRNA expression occurred within 6 hours, consistent with CTGF being an immediate early gene. However, the interaction between FSH and TGF $\beta$ -1 was not observed until 48 hours, suggesting that TGF $\beta$ -1 acts by increasing FSH receptors. GDF-9 and activin-A were also stimulatory, and their actions were inhibited by FSH.

CTGF gene expression is upregulated by conditions favouring cell replication, but downregulated when differentiation towards oestrogen biosynthesis is favoured. Given this orchestrated regulation of CTGF mRNA, we predict that the encoded protein will have crucial roles in ECM deposition and tissue remodelling in the developing follicle.

1 **Slee RB**, Hillier SG, Lague P, *et al.* Differentiation-dependent expression of connective tissue growth factor and lysyl oxidase messenger ribonucleic acids in rat granulosa cells. *Endocrinology* 2001;**142**:1082–9.

### 03 TWO NOVEL CIS-ACTING ELEMENTS OF HUMAN CTGF/CCN2 GENE EXPRESSION

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The connective tissue growth factor (ctgf/ccn2) gene is one of the prototypic members of the CCN family. It is a multifunctional growth factor that mediates the cell growth and differentiation of a variety of mesenchymal cell species including chondrocytes. Physiologically and pathologically, ctgf/ccn2 gene expression is strictly controlled and highly restricted, which indicates that it is under the control of complex gene regulation machinery.

We identified a novel post-transcriptional regulatory RNA element, a cis-acting element for structure-anchored repression (CAESAR) in the 3'-untranslated region of the ctgf/ccn2 gene.<sup>2</sup> CAESAR is known to form a stable secondary structure in vitro, and its cellular binding counterpart is also suggested. The structural requirement of CAESAR in its repressive function was dissected, and we found that multiple stem-loop structures were required for the function of CAESAR, rather than internal bulge and stable major stem-loop structures. In addition, the functioning stage of CAESAR after transcription was analyzed. Subcellular fractionation and ribonuclease protection analysis of a CAESAR-containing mRNA revealed no significant effect of CAESAR on either stability or nuclear export of the mRNA. Nevertheless, in vitro translation analysis disclosed the attenuation of translation efficiency caused by the CAESAR linked in cis. These findings indicate that CAESAR represses gene expression, mainly by affecting the translation efficiency of the mRNA.

Most recently, another cis-element was discovered in the promoter region of ctgf/ccn2. Since this novel element is thought to be involved in chondrocyte-specific induction of gene expression, it was entitled TRENDIC—a transcriptional enhancer dominant in chondrocytes.

Mutational analysis of TRENDIC, which is as long as approximately 20 bp, revealed that point mutations abolished the strong activity of the *ctgf/ccn2* promoter in chondrocytic HCS-2/8 cells. Certain nuclear factor(s) were found to bind to TRENDIC, which was, interestingly, observed in a chondrocyte-specific manner. Finally, we found that insertion of a few TRENDIC copies adjacent to the SV40 promoter drastically enhanced the marker gene expression driven by this promoter. The above results revealed that the strict regulation of *ctgf/ccn2* gene expression is enabled by the combination of a variety of cis-elements, including the two elements described herein.

2 **Kubota S**, Kondo S, Eguchi T, *et al.* Identification of an RNA element that confers post-translational repression of connective tissue growth factor/hypertrophic chondrocyte specific 24 (*ctgf/hcs24*) gene: similarities to retroviral RNA-protein interactions. *Oncogene* 2000;19:4773–86.

#### 04 MODULATION OF CTGF GENE EXPRESSION BY ALTERATIONS OF THE CYTOSKELETAL ARCHITECTURE

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Expression levels of CTGF were shown to be modulated by static pressure or shear stress, suggesting that changes in cellular morphology regulate CTGF expression. Therefore, we investigated different aspects of cytoskeletal alteration and their implications for the induction of CTGF in renal mesangial cells and fibroblasts.

(1) Induction of CTGF by exogenous stimuli was dependent on an intact actin cytoskeleton. Pre-treatment of the renal cells with cytochalasin D or latrunculin B prevented induction by transforming growth factor  $\beta$  (TGF- $\beta$ ) and other stimuli.

(2) Disruption of actin stress fibres by cytochalasin D, but not by latrunculin B, led to a transient increase of CTGF mRNA expression with peak levels after about 1 hour. The different molecular mechanisms of the F-actin disrupting agents were visualised by staining actin stress fibres: cytochalasin D induced a patchy actin filament pattern, whereas latrunculin B led to a gradual disappearance of the actin fibres. The increase in CTGF mRNA levels by cytochalasin D was inhibited by incubation with actinomycin D, indicative of transcriptional regulation. The transcription factors sensing the cytochalasin D-mediated changes in the actin cytoskeleton and the targeted response elements remain to be identified.

(3) A more pronounced induction of CTGF mRNA and protein was obtained by interference with the microtubular system by nocodazole or colchicine. Pre-incubation of the cells with Y27632, an inhibitor of RhoA-associated kinases, interfered with colchicine-mediated CTGF induction, indicative of an activation of RhoA by disruption of the microtubular system.

Taken together, our data support an essential role for the small GTPase RhoA, not only for the induction of CTGF by exogenous stimulatory proteins, but also as a molecular switch, translating changes in cell morphology into gene expression.

#### 05 RETINOID SIGNALING INDUCES CONNECTIVE TISSUE GROWTH FACTOR IN MATURING CHONDROCYTES AND ACTS THROUGH THE ERK 1/2 MAP KINASE PATHWAY

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Connective tissue growth factor (CTGF) participates in chondrocyte proliferation, maturation and hypertrophy during endochondral bone formation, but the mechanisms regulating its expression and action are far from clear. Because retinoids and their nuclear receptors are required for chondrocyte maturation and ossification, we asked whether and how retinoids may regulate CTGF expression. Homogenous populations of chondrocytes at advanced stages of maturation were isolated from the cephalic portion of chick embryo sterna and reared in high density monolayer cultures. Cultures were then treated with physiological doses (10–100 nM) of all-trans-retinoic acid (RA) for several days; for comparison, parallel cultures were treated with other positive or negative modulators of chondrocyte maturation, including transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1), bone

morphogenetic protein-2 (BMP-2) or parathyroid hormone-related peptide (PTHrP). RA treatment markedly upregulated CTGF gene expression and did so in a dose- and time-dependent manner. CTGF expression was also stimulated by TGF- $\beta$ 1 and BMP-2, but inhibited by PTHrP. CTGF upregulation by RA was prevented by co-treatment with the synthetic retinoid antagonist Ro 41–5253, attesting to the specificity of RA action. When used in the absence of RA, the antagonist reduced baseline expression of CTGF in the cells and even blocked the effects of exogenous recombinant CTGF on chondrocyte maturation. To analyze the mechanisms of RA action, cultures were treated with various inhibitors. RA induction of CTGF expression was inhibited by co-treatment with cycloheximide or the MEK 1/2 inhibitor PD 98059. In contrast, when the cells were treated with the p38 pathway inhibitor SB 203580, CTGF gene expression was stimulated. Lastly, RA treatment led to activation of ERK 1/2 and p38 in a time- and dose-dependent manner. Our results show that CTGF expression during chondrocytes maturation is positively influenced by retinoid signaling and the ERK 1/2 mitogen activated protein kinase (MAPK) pathway, but is inhibited by activation of p38. Thus, a balance among ERK 1/2 and p38 MAPK pathways and retinoid signaling appears to be crucial in regulating CTGF expression and action in maturing and hypertrophic chondrocytes.

#### 06 CCN5 IS A GROWTH ARREST-SPECIFIC GENE THAT REGULATES SMOOTH MUSCLE CELL PROLIFERATION AND MOTILITY

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Vascular smooth muscle cell (VSMC) hyperplasia plays an important role in both chronic and acute vascular pathologies. Considerable work has focused on the mechanisms regulating VSMC growth and the search for agents that could suppress VSMC proliferation and motility. One of the inhibitors studied is the glycosaminoglycan heparin. Using a subtractive hybridization approach, we isolated and characterized a novel growth arrest-specific (*gas*) gene induced in VSMC exposed to heparin.<sup>3,4</sup> This gene is a member of the CCN family—CCN5.

Using an adenoviral vector to express CCN5, we provide functional evidence that this protein can inhibit VSMC proliferation, motility, and invasiveness. In contrast, VSMC adhesion and apoptosis are unaffected by CCN5 overexpression. Because we have shown that CCN5 is localized to the cell surface and extracellular matrix of VSMC, we are examining the effects of CCN5 on matrix and matrix-regulating molecules. Our results indicate that MMP-2 protein levels are inhibited by CCN5 in these cells. We also significantly extend previous data from our laboratory suggesting that CCN5 is a *gas* gene, and map for the first time the localization of CCN5 protein in balloon injured rat carotid arteries. These data suggest that CCN5 is temporally and spatially expressed in a manner consistent with a role in regulating proliferation, motility, and invasiveness of VSMC in vivo and in vitro.

3 **Delmolino LM**, Castellot JJ, Jr. Heparin suppresses *sgk*, an early response gene in proliferating vascular smooth muscle cells. *J Cell Physiol* 1997;173:371–9.

4 **Delmolino LM**, Stearns NA, Castellot JJ, Jr. COP-1, a member of the CCN family, is a heparin-induced growth arrest specific gene in vascular smooth muscle cells. *J Cell Physiol* 2001;188:45–55.

#### 07 DIFFERENTIAL EXPRESSION OF CCN-FAMILY MEMBERS IN PRIMARY HUMAN MESENCHYMAL STEM CELLS FROM BONE MARROW DURING DIFFERENTIATION INTO OSTEOBLASTS, CHONDROCYTES AND ADIPOCYTES

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**Introduction:** Members of the CCN-family of connective tissue growth factors (CTGF), the cystein-rich protein 61 (CYR61) and the nephroblastoma overexpressed protein (nov) function in processes such as differentiation and tissue regeneration mainly as growth factors and matrix associated signal molecules. Previously, we have shown that the expression of human CYR61 (hCYR61) in vivo was associated with conditions of enhanced bone formation and fracture healing. Further results in the literature point towards the expression of other CCN proteins in bone such as CTGF, CTGF-L and WISP-3.

**Methods:** Primary cultures of human mesenchymal stem cells were derived from the femoral head of patients undergoing total hip arthroplasty. Differentiation into adipocytes and osteoblasts was achieved in monolayer culture, differentiation into chondrocytes was induced in pellet culture. For either pathway, established differentiation markers and CCN-members were analyzed at the mRNA level by reverse transcription polymerase chain reaction (RT-PCR) and at the protein level by immunocytochemistry.

**Results:** RNA or protein levels of established markers revealed the appropriate phenotype of differentiated cells (alizarin-red, alkaline phosphatase, osteocalcin, collagens, cbfa1, PPAR $\gamma$  (peroxisome proliferative activated receptor  $\gamma$ ), oil-red-o, aggrecan). Mesenchymal stem cells expressed nestin mRNA and all CCN-family mRNAs. The expression of hCYR61 was high in stem cells and decreased 7-fold during osteogenesis and chondrogenesis and more than 10-fold during adipogenesis. These results were confirmed by immunocytochemical analyses. CTGF-L and WISP-3 RNA expression were enhanced slightly during osteogenesis and decreased in chondrogenesis and adipogenesis (3- and 10-fold, respectively). CTGF-L expression was decreased > 10-fold in adipogenesis.

**Discussion:** The expression of CCN-family members was dependent on the differentiation status of human mesenchymal stem cells in vitro. hCYR61, which plays a role as a signalling molecule of the extracellular matrix, is important in the early nestin positive stem cells used in this study. The marked decrease in hCYR61 expression during all differentiation pathways suggests a specific role of hCYR61 for maintenance of the stem cell phenotype. The differential expression of WISP-3, CTGF, CTGF-L and mainly hCYR61 indicates that the CCN-family might be important for the function of mesenchymal stem cells as well as the regulation of proliferation and specific differentiation pathways of human mesenchymal stem cells from bone marrow.

#### 08 COMPARATIVE ANALYSIS OF SPATIOTEMPORAL EXPRESSION OF SEVERAL CCN GENES IN CHICK EMBRYO

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Control of cell proliferation and differentiation requires the interplay of many signaling factors. The emerging family of proteins, often referred to as CCN, sharing a common multimodular organization, includes both positive and negative regulators of cell growth. Among this gene family, the first three of them (CCN1, CCN2 and CCN3) would be expressed early in development. Comparing their expression at different stages of development in chick embryos would help to determine the importance of these genes in morphogenesis and differentiation during embryonic development. In situ hybridization has been used to obtain spatiotemporal patterns of CCN gene expression. Olfactory structure has been included because it is the site of neurogenesis both during embryonic development and in the adult. The comparison of CCN gene expression during embryogenesis should provide information about their roles in the formation of different organs and should also reveal possible interactions between the CCN proteins in these processes.

#### 09 CTGF INTERACTIONS WITH HEPATIC STELLATE CELLS

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Fibrosis of major organs is a serious health problem world-wide, yet the underlying molecular mechanisms require further investigation and effective therapies need to be developed. Fibrosis is characterized by inappropriate proliferation of mesenchymal cells and the accumulation of extracellular matrix (ECM), which leads to impaired function and ultimate destruction of normal organ architecture. CTGF acts to promote fibroblast proliferation, migration, adhesion, and ECM formation, and its over-production is proposed to play a major role in pathways that lead to fibrosis, especially those that are transforming growth factor  $\beta$  (TGF- $\beta$ )-dependent. The link with TGF- $\beta$  has arisen from many studies showing that CTGF is transcriptionally activated by TGF- $\beta$ , that both CTGF and TGF- $\beta$  are pro-fibrogenic, and that they are co-expressed in numerous fibrotic lesions. Studies from several laboratories support a strong link between CTGF production and TGF- $\beta$  action in hepatic fibrosis. In humans, it has previously been shown that patients with biliary atresia have higher circulating levels of CTGF than their normal counterparts and that human fibrotic liver from

patients with primary biliary cirrhosis, primary sclerosing cholangitis, or biliary atresia exhibit high levels of CTGF. CTGF expression is also raised in experimentally induced liver fibrosis in rats following CCl<sub>4</sub> administration or bile duct ligation and is correlated with enhanced levels of TGF- $\beta$  or collagen.

Of the cells that overexpress CTGF in liver fibrosis, there is considerable interest in hepatic stellate cells (HSCs), which undergo activation and transition from quiescent vitamin A-rich cells to vitamin A-deficient, proliferative, fibrogenic, contractile myofibroblasts. In view of recent reports demonstrating that CTGF is expressed during HSC activation or in response to TGF- $\beta$ , we have started to study the production and action of CTGF by HSCs in vitro. Our data support a role for 38 kDa CTGF and various C-terminal CTGF isoforms (20 kDa, 10 kDa) in stimulating certain HSC functions, including cell adhesion, smooth muscle actin expression, and activation of intracellular signaling pathways. The adhesive properties of CTGF are heparin-dependent but do not appear to involve integrins. The identification of additional CTGF-regulated genes in HSCs is currently being addressed by gene array technology. Furthermore, we have found that the pattern of cellular expression of CTGF in human fibrotic liver varies according to the nature of the disease process, suggesting that a broader view is needed regarding the biology of CTGF in liver fibrosis.

#### 10 THE NOV (CCN3) PROTEIN: CROSSROADS OF DIFFERENT SIGNALING PATHWAYS?

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An increasing body of evidence suggests that NOV and other CCN proteins are involved in fundamental processes governing cell proliferation and differentiation.

Immunocytochemical detection of the NOV protein in the developing human, rat and chicken embryo has indicated that it is associated with the differentiation of several tissues originating from the three germ layers. Major sites of expression in normal conditions included the nervous system (axons and neurons), adrenal, striated and cardiac muscle, cartilage and bone. Studies aimed at establishing whether nov expression was altered in tumors originating from these tissues found that the detection of nov in tumor tissues can have a good or bad prognosis, depending upon the origin, type and stage of the tumor samples. Understanding the biological basis for these differences requires a better knowledge of the role of NOV in normal tissues.

Our current working model is based on the assumption that the biological properties of NOV, and other CCN proteins, are dependent upon their interactions with partners that may be either positive or negative effectors in cell growth control. Along this line, the bioavailability of both the CCN proteins and their respective partners would increase the variety of biological responses. Identifying these potential partners should permit us to get a better insight into the nature of the signaling pathways in which NOV is acting.

To address this problem, we have first undertaken a screening of three different yeast two-hybrid libraries. Among the different clones that proved to interact physically with NOV, most of them turned out to be signaling proteins and, in some cases, recent data have confirmed that NOV was indeed involved in the corresponding signaling pathways. Preliminary results also suggest that CTGF (CCN2) interacts with some NOV partners.

More recent data have indicated that NOV also interacts with calcium binding proteins and is able to modulate the intracellular concentration of calcium ions. These observations assigned, for the first time, a biological function to NOV and provided a clue for the detection of NOV at sites where calcium ions are known to play critical roles.

The biological significance of these various interactions and the potential role of NOV in signaling will be discussed in the light of the results that have been obtained in both normal and pathological conditions.

#### 11 CYR61 IS AN ONCOGENE IN GLIOMAS THAT STIMULATES THE INTEGRIN LINKED KINASE MEDIATED B-CATENIN-TCF/LEF AND AKT SIGNALING PATHWAYS

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Cyr61 is a member of the CCN family of growth factors; these proteins are secreted and can act as ligands of distinct integrins. We

show that Cyr61 can behave as an oncogene in gliomas, acting through activated integrin-linked kinase (ILK) to stimulate  $\beta$ -catenin-TCF/Lef and Akt signaling pathways. Overexpression of Cyr61 occurred in highly tumorigenic glioma cell lines, as well as 68% of the most malignant glioblastoma multiforme (GBM) brain tumors. Forced expression of Cyr61 in U343 glioma cells accelerated their growth in liquid culture and enhanced their anchorage-independent proliferation in soft agar, as well as significantly increasing their ability to form large, vascularized tumors in nude mice. Overexpression of Cyr61 in the U343 cells led to the upregulation of distinct integrins, including  $\beta$ 1 and  $\alpha$ v $\beta$ 3, which have been shown to interact with both Cyr61 and ILK. The activity of ILK was dramatically increased in these cells. Overexpression of Cyr61 also resulted in the phosphorylation of GSK3 $\beta$ , as well as accumulation and nuclear translocation of  $\beta$ -catenin, leading to activation of the  $\beta$ -catenin-TCF/Lef signaling pathway. Furthermore, forced expression of Cyr61 in the glioma cells activated the PI3 kinase pathway, resulting in prominent phosphorylation of both Akt and the antiapoptotic protein Bad. Cyr61 appears to stimulate several signaling pathways in the development of gliomas.

### 12 CONNECTIVE TISSUE GROWTH FACTOR MEDIATES TGF $\beta$ -DEPENDENT CELL CYCLE ARREST IN THE G1 PHASE IN MESANGIAL CELLS

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Transforming growth factor  $\beta$  (TGF- $\beta$ ) causes renal mesangial cells to arrest in the G1 phase of the cell cycle by blocking the transition from G1 to S phase. This has been shown to induce cellular hypertrophy in mesangial cells—one of the earliest abnormalities of diabetic nephropathy. The molecular mechanisms underlying this G1 phase arrest appear to result from the transcriptional activation of the cyclin dependent kinase inhibitors (CDKIs), p15INK4 and p21Cip1, which are known to bind and inactivate cyclin D-CDK4/6 and the cyclin E-CDK2 kinase complexes. This in turn causes the maintenance of pRb protein in a very low phosphorylation state, preventing cell cycle progression. In our laboratory we found that CTGF can also promote mesangial cell cycle arrest in the G1 phase, through the same molecular mechanisms. Interestingly, using CTGF antisense oligonucleotides, our results indicate that CTGF mediates the TGF- $\beta$ -induced cell cycle arrest and hypertrophy of these cells. The molecular mechanism by which CTGF regulates the TGF- $\beta$  signalling pathway will be discussed.

### 13 WISP3, A TUMOR SUPPRESSOR GENE OF INFLAMMATORY BREAST CANCER BINDS TO IGF-I AND MAY MODULATE IGF SIGNALING

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**Introduction:** Inflammatory breast cancer (IBC) is the most lethal form of locally advanced breast cancer. In our previous work, we found that WISP3 is lost in 80% of human IBC tumors and that it has growth and angiogenesis inhibitory functions in breast cancer in vitro and in vivo. WISP3 is a CCN protein with a multimodular structure with an insulin-like growth factor binding protein (IGFBP) motif. The role of this highly conserved domain in the function of WISP3 is unknown. Insulin-like growth factor (IGF) signaling is a strong predictor of mammographic density and breast cancer risk in premenopausal women. We hypothesize that WISP3 may exert its tumor inhibitory function in IBC at least in part by binding to IGF-I and/or binding to the IGF-I receptor (IGF-IR) through its conserved IGFBP domain, and thus modulating IGF signaling.

**Methods:** To test whether WISP3 binds to IGF-I we performed a competitive binding assay in solution using recombinant WISP3 protein and recombinant labeled and unlabeled IGF-I. The effect of WISP3 on IGF-IR activation was studied in SUM149 cells stably transfected with WISP3. These cells derive from a highly aggressive primary inflammatory breast cancer and have lost WISP3 in their wild-type state. The IGF-IR was immunoprecipitated from protein lysates of SUM149 cells transfected with WISP3 and controls. Tyrosine phosphorylation was assessed by western blot with an anti-phosphotyrosine monoclonal antibody. To study the cellular localization of WISP3, we performed immunofluorescence on transiently transfected SUM149 cells with WISP3 labeled with green fluorescent protein (GFP).

**Results:** WISP3 competitively binds to IGF-I in solution. Biotinylated IGF-I and unlabeled IGF-I competed for WISP3 binding. We show that WISP3 markedly inhibits IGF-IR activation when compared with

controls. By immunofluorescence, WISP3 localizes to the cytoplasm of the breast epithelial cells.

**Conclusion:** WISP3 competitively binds to IGF-I and drastically inhibits IGF-IR activation, which may explain at least some of the growth inhibitory effects of WISP3 in inflammatory breast cancer.

### 14 CTGF-DEPENDENT BINDING AND SIGNALING TO DIVERSE CELL TYPES

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We have investigated the biological effects of CTGF in cell culture in an attempt to understand more fully the function of CTGF and its mechanism of action. This has involved the development of several useful systems to study CTGF activity, namely: (1) a CTGF-dependent cell adhesion and survival assay; (2) a CTGF-dependent signaling assay; and, (3) a CTGF cell-binding assay. We have examined the role of integrins in these experimental systems and compared the potencies of native and recombinant human CTGFs and CTGF fragments. These studies have shown distinct biochemical properties for the different sourced CTGFs and also demonstrate that the behavior of many cell types, including leukocytes, can be influenced by CTGF.

Monoclonal antibodies that recognize specific epitopes in different regions of the CTGF molecule have allowed expansion of work with the CTGF fragments so that structure and function can be correlated more precisely. We have used this approach to identify and define the CTGF domains and structures that are essential for mediating the CTGF activities demonstrated in bioassays. Distinct differences were observed between the bioactivity of purified N-terminal and C-terminal halves of CTGF, and in some cases full length CTGF could be maintained in a cryptic conformation that may relate to its functional activity in vivo.

### 15 CCN PROTEINS ARE NOVEL LIGANDS OF INTEGRIN $\alpha$ M $\beta$ 2 ON PERIPHERAL BLOOD MONOCYTES

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Cysteine-rich 61 (Cyr61; CCN1) and connective tissue growth factor (CTGF; CCN2) are growth factor-inducible immediate-early gene products found in atherosclerotic lesions, restenosed blood vessel walls and healing cutaneous wounds. We previously reported that the adhesion of endothelial cells, platelets and fibroblasts to these CCN proteins is mediated through integrin receptors. Because monocyte adhesion and transmigration are important for atherosclerosis, inflammation, and wound healing, we examined monocyte interactions with Cyr61 and CTGF. Isolated peripheral blood monocytes adhere to Cyr61- and CTGF-coated wells in an activation- and divalent cation-dependent manner.

Furthermore, monocyte adhesion to Cyr61 is specifically blocked by monoclonal antibodies directed against the integrin  $\alpha$ M or  $\beta$ 2 subunit, indicating that integrin  $\alpha$ M  $\beta$ 2 serves as an adhesion receptor on monocytes for these CCN proteins. Consistently, a glutathione-S-transferase (GST)-fusion protein containing the I domain of the  $\alpha$ M subunit binds specifically to immobilized Cyr61 and CTGF. Using synthetic peptides encompassing partial sequences of Cyr61, we identified a unique 13-residue sequence in Cyr61 that supports  $\alpha$ M  $\beta$ 2-mediated monocyte adhesion and GST- $\alpha$ M I domain fusion protein binding. To examine the functional significance of monocyte adhesion to Cyr61, we performed northern analysis and cDNA array to identify genes regulated by this adhesion process.

Thus, monocyte adhesion to Cyr61 induces the expression of inflammatory mediators including interleukin 1 $\beta$  (IL-1 $\beta$ ), IL-8, monocyte chemoattractant protein 1, myeloid related protein 8 (MRP8) and MRP14. Together, these findings indicate that Cyr61 and CTGF are novel ligands of integrin  $\alpha$ M  $\beta$ 2 on monocytes, and suggest an important pathophysiological role of monocyte adhesion to these CCN proteins.

### 16 INTEGRIN-MEDIATED FUNCTIONS OF CYR61: A STRUCTURAL ANALYSIS

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CYR61 and CTGF are non-RGD-containing ligands of integrin receptors, which bind directly to various integrins including  $\alpha$ <sub>v</sub> $\beta$ <sub>3</sub>,  $\alpha$ <sub>v</sub> $\beta$ <sub>5</sub>,  $\alpha$ <sub>6</sub> $\beta$ <sub>1</sub>,

$\alpha_{\text{IIb}}\beta_3$ , and  $\alpha_{\text{v}}\beta_3$ . At least some of the activities of CYR61 and CTGF can be attributed to signaling through their interaction with integrin receptors. Through extensive characterization of integrin utilization by CYR61 in various cell types, we show that CYR61 interacts with distinct integrins in a cell type- and function-specific manner to carry out disparate biological activities. Thus, cell adhesion to CYR61 in platelets and monocytes is mediated through integrins  $\alpha_{\text{IIb}}\beta_3$  and  $\alpha_{\text{v}}\beta_3$ , respectively, whereas cell adhesion in fibroblasts and smooth muscle cells is accomplished via integrin  $\alpha_v\beta_1$  and heparan sulfate proteoglycans, respectively. CYR61 stimulated chemotaxis in fibroblasts, smooth muscle cells, and endothelial cells is mediated through integrins  $\alpha_3\beta_1$ ,  $\alpha_v\beta_1$ , and  $\alpha_3\beta_3$ , respectively. The mitogenesis enhancing activity of CYR61, on the other hand, is dependent upon interaction with integrin  $\alpha_3\beta_3$  in both fibroblasts and endothelial cells. Furthermore, CYR61 selectively interacts with integrins  $\alpha_3\beta_1$  and  $\alpha_v\beta_1$  to mediate angiogenic activities in endothelial cells, depending on the activation state of the cell.

We have used mutational analysis to dissect the structural elements required for CYR61 function. The C-terminal cysteine knot (CT) domain contains heparin-binding sites, and mutations at these sites abolish heparin-binding activity. The heparin-binding defective mutants are unable to support fibroblast adhesion, but maintain the ability to support  $\alpha_3\beta_3$ -mediated endothelial cell adhesion. Deletion of the entire C-terminal CT domain of CYR61 revealed that the CT domain is dispensable for chemotactic and mitogenesis enhancing activities in fibroblasts but is essential for supporting cell adhesion. Domain-specific antibodies have been used to map regions of CYR61 required for various activities. Furthermore, we are using a synthetic peptide library of CYR61 to identify integrin binding sites. Together, results of these studies have begun to delineate a structural map for CYR61 functions.

#### 17 THE INTEGRIN $\alpha_v\beta_5$ MEDIATES OSTEOBLAST ADHESION TO CONNECTIVE TISSUE GROWTH FACTOR

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Connective tissue growth factor (CTGF) is a secreted, extracellular matrix-associated signaling protein that regulates diverse cellular functions including adhesion, proliferation and differentiation. We recently showed that osteoblasts in situ express CTGF during active growth of the skeleton, as well as during fracture repair. Primary osteoblast cultures demonstrated a bimodal pattern of CTGF expression, which was relatively high during the period of proliferation and reached peak levels during the period of mineralization. Furthermore, blocking the activity of the endogenous CTGF produced and secreted in these cultures using an anti-CTGF antibody caused a dramatic dose-dependent inhibition of nodule formation and mineralization. Other laboratories have shown that specific integrins serve as receptors for the adhesion of fibroblasts, endothelial cells and platelets to CTGF. In this study, we examined whether MC3T3-E1 osteoblastic cells can adhere to an immobilized CTGF substrate and determined whether specific integrins are involved in this adhesive interaction. MC3T3-E1 cells were plated into culture wells pre-coated with increasing concentrations of recombinant CTGF (rCTGF) with 1% bovine serum albumin (BSA) serving as a negative control and fibronectin serving as a positive control. MC3T3-E1 cells adhered to rCTGF-coated wells in a dose-dependent manner, with maximal adherence beginning at 5  $\mu\text{g}/\text{ml}$ . We then examined whether specific integrins mediate this adhesive interaction by pre-incubating the MC3T3-E1 cells with a panel of anti-integrin antibodies including anti- $\alpha_3\beta_1$ , anti- $\alpha_{\text{IIb}}\beta_3$ , and anti- $\alpha_v\beta_5$ . The anti- $\alpha_v\beta_5$  antibody decreased cell adhesion by as much as 70%, while other antibodies, such as the anti- $\alpha_3\beta_1$  antibody, had little or no effect on cell adhesion. These data suggest that the integrin  $\alpha_v\beta_5$  mediates the adhesion of MC3T3-E1 osteoblastic cells to rCTGF. Next, we examined the formation of focal adhesions and focal adhesion kinase (FAK) activation in MC3T3-E1 cells adhered to rCTGF. Immunofluorescent analyses showed that paxillin is localized at the ends of actin stress fibers, suggesting the formation of focal adhesions. Furthermore, clusters of  $\alpha_v\beta_5$  integrin co-localized with paxillin at sites of focal adhesion, and activated (phosphorylated) FAK also localized to focal adhesions. As a result of adhesion to rCTGF, osteoblasts exhibited reorganization of the actin cytoskeleton and the formation of cellular protrusions (microspikes and lamellipodia) consistent with cell spreading and migration. The induction of distinct signaling pathways mediated by this CTGF-integrin interaction may provide a molecular basis for some of the effects of CTGF on osteoblast differentiation and function.

#### 18 NOV (NEPHROBLASTOMA OVEREXPRESSED GENE, CCN3) PROTEIN ASSOCIATES WITH NOTCH1 EXTRACELLULAR DOMAIN AND INHIBITS MYOBLAST DIFFERENTIATION VIA NOTCH SIGNALING PATHWAY

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We demonstrate a novel interaction of NOV, a member of the CCN gene family, with the Notch signaling pathway. NOV associates with the epidermal growth factor (EGF)-like repeats of Notch1 by the C-terminal cysteine knot (CT) domain. The promoters of HES1 and HES5, which are the downstream transducers of Notch signaling, were activated by NOV. The expression of NOV and Notch1 was concomitant in the presomitic mesoderm and later in myocytes and chondrocytes, suggesting that they have a synergistic effect in mesenchymal cell differentiation. In C2/4 myogenic cells, increased expression of NOV led to downregulation of MyoD and myogenin, resulting in inhibition of myotube formation. These results indicate that NOV-Notch1 association exerts a positive effect on Notch signaling and consequently suppresses myogenesis.

#### 19 NOV-NOTCH ASSOCIATION AND ITS BIOLOGICAL MEANING DURING CELL DIFFERENTIATION

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Nov (ccn3) expression was investigated at different developmental stages. Nov expression became visible when the regression of the Hensen's node started (Hamburger and Hamilton stage (HH) 5+) at gastrulation. Expression was strong in the notochord forming area, which shows a left-right asymmetrical pattern. The left deviated pattern was similar to that of sonic hedgehog (shh), but the expression area was more restricted than that seen for shh. Expression in the notochord forming area continued until the end of gastrulation (HH20); however, left-right asymmetry disappeared at HH8.

From HH7, expression in the presomitic mesoderm (psm) was also visible. A psm specific expression pattern is specific to the notch signaling genes and we investigated the relationship of Nov with them. Immunoprecipitation-western blot assay experiments revealed the discrete binding of Nov to Notch1. Detailed analysis showed that the Notch1 extracellular epidermal growth factor (EGF) domain was recognized by the Nov cysteine knot (CT) domain. This binding influenced the activity of the notch signal (HES promoter activity) and delayed myogenesis in the culture system.

The expression pattern of notch signal genes was investigated at gastrulation and only Serrate1 (Jagged1), a ligand of Notch1, expression showed a left-right asymmetrical pattern. We are investigating the Nov-notch association in the early stage of development.

#### 20 THE ROLE OF WISPS IN CHONDROCYTE DIFFERENTIATION

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**Introduction and aims:** Wisp genes (Wnt-inducible secreted proteins) are members of the CCN family of growth factors, which includes connective tissue growth factor (CTGF) and Cyr61. Mutations in the human Wisp3 gene cause progressive pseudorheumatoid dysplasia (PPD), a disease characterised by loss of articular cartilage and joint space, with an abnormal arrangement of chondrocytes in the growth plate. It is therefore likely that Wisps play important roles during chondrogenesis. Furthermore, in the mammary epithelial cell line C57MG, Wisp1 and Wisp2 are upregulated by Wnt1 signalling. Wisp1 is also directly activated by  $\beta$ -catenin and can partially mimic the actions of Wnt1. Recent evidence has shown that Wnts participate in chondrogenesis; thus, it is possible that Wisps mediate the function

of Wnts during chondrogenesis. Therefore, we have investigated the expression and function of Wisp genes in the developing chick limb.

**Methods:** Chick homologues of Wisp genes were cloned by screening a stage 12 whole embryo cDNA library and by degenerate reverse transcription polymerase chain reaction (RT-PCR) using RNA isolated from stage 28 humerus cartilage. Following this, Wisp expression was mapped by *in situ* hybridisation on tissue sections of the developing chick limb. To investigate the function of Wisps in chondrogenesis, full-length Wisp1 and Wisp3 cDNAs were overexpressed in micromass cultures using the chick replication competent retrovirus, RCAS (BP).

**Results:** cDNA fragments encoding cWisp1 and cWisp3 were isolated by degenerate RT-PCR and library screening, respectively. *In situ* hybridisation revealed that cWisp1 is expressed in a subset of prehypertrophic chondrocytes between stages 28 and 30 in the chick wing. Between stages 30 and 37, cWisp1 expression is no longer within the cartilage element itself, but becomes localised to the perichondrium and the joint. Eventually, in the stage 40 tibiotarsus, cWisp1 becomes restricted to the subchondral and cortical bone. To date, we have been unable to detect cWisp3 by this method; however, RT-PCR has demonstrated that cWisp3 is expressed at low levels in the cartilage from stage 28 and 32 humeri. The functional studies have revealed that Wisp3 subtly yet significantly increases chondrogenesis in micromass cultures. In contrast, Wisp1 seems to have a small negative effect on chondrogenesis.

**Conclusions:** Wisp1 and Wisp3 are expressed in developing chick cartilage and appear to exhibit opposing effects on chondrocyte differentiation. Studies are currently in progress to delineate Wisp function during chondrogenesis and to understand the signalling pathway from Wnts to Wisps.

## 21 XENOPUS CYR61 IS A MATERNAL GENE PRODUCT THAT MODULATES WNT SIGNALLING AND REGULATES GASTRULATION

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Cyr61 is a secreted, heparin-binding, extracellular matrix-associated protein whose functions include the promotion of adhesion and chemotaxis and the stimulation of growth of fibroblasts and endothelial cells.

We have cloned and characterised xenopus Cyr61 (Xcyr61). Overexpression studies and the use of antisense morpholino oligonucleotides indicate that Xcyr61 plays a role in the regulation of gastrulation.

This role might be mediated through its adhesive activities and perhaps also through its remarkable ability, in a context-dependent manner, either to stimulate or to inhibit Wnt signalling.

Further details will be presented at the meeting.

## 22 CYR61 (CCN1) IS ESSENTIAL FOR PLACENTAL DEVELOPMENT AND VASCULAR INTEGRITY

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CYR61 (CCN1) is a member of the CCN family of secreted proteins, which includes connective tissue growth factor (CTGF; CCN2), NOV (CCN3), WISP-1 (CCN4), WISP-2 (CCN5), and WISP-3 (CCN6). First identified as the product of a growth factor inducible immediate-early gene, CYR61 is an extracellular matrix-associated angiogenic inducer that functions as a ligand of integrin receptors to promote cell adhesion, migration, and proliferation. Aberrant expression of Cyr61 is associated with breast cancer, wound healing, and vascular diseases such as atherosclerosis and restenosis. To understand the functions of CYR61 during development, we have disrupted the Cyr61 gene in mice. We show here that Cyr61-null mice suffer embryonic death due to failure of chorioallantoic fusion, placental vascular insufficiency, and compromised vessel integrity, thus establishing CYR61 as a novel and essential regulator of vascular development. CYR61 deficiency results in a specific defect in vessel bifurcation (non-sprouting angiogenesis) at the chorioallantoic junction, leading

to an undervascularized placenta without affecting differentiation of labyrinthine syncytiotrophoblasts. This unique phenotype is correlated with impaired VEGF-C expression in the allantoic mesoderm, suggesting that CYR61 regulated expression of VEGF-C plays a role in vessel bifurcation. The genetic and molecular basis of vessel bifurcation is currently unknown, and these findings provide new insight into this aspect of angiogenesis.

## 23 CONNECTIVE TISSUE GROWTH FACTOR COORDINATES CHONDROGENESIS AND ANGIOGENESIS DURING SKELETAL DEVELOPMENT

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Production and remodeling of the extracellular matrix is an essential process during the development of most tissues, yet little is known about how this is accomplished. A role for the secreted protein connective tissue growth factor (CTGF; CCN2) is suggested by its ability to mediate collagen deposition during fibrotic responses. CTGF is induced by, and is required for transforming growth factor  $\beta$  (TGF $\beta$ ) to promote excessive collagen production in a wide variety of fibrotic diseases. The profound effects of CTGF as a mediator of fibrosis suggest an essential role in extracellular matrix production in normal tissues. To test this, we examined the pattern of Ctgf expression and found that Ctgf and Tgf $\beta$  are coexpressed in chondrocytes, with highest levels in hypertrophic cartilage. To test whether CTGF cooperates with TGF $\beta$  during chondrogenesis, we performed gain-of-function experiments using CTGF-expressing retroviruses. CTGF stimulates chondrocytic cell proliferation, and collaborates with TGF $\beta$  to induce extracellular matrix (ECM) synthesis. We confirmed that CTGF is a crucial regulator of ECM content by generating Ctgf<sup>-/-</sup> mice. Ctgf deficiency leads to multiple skeletal dysmorphisms due to defective chondrocyte proliferation and to decreased synthesis of specific extracellular matrix components. Unexpectedly, we found that the hypertrophic zones of long bones of Ctgf<sup>-/-</sup> mice are expanded as a result of impaired ECM remodeling, resulting from loss of matrix metalloproteinases. Moreover, expression of vascular endothelial growth factor (VEGF) at the growth plate is reduced in Ctgf mutants, and growth plate angiogenesis is blocked as a consequence. These results demonstrate that CTGF is required for chondrogenesis during development, and is a key regulator coupling ECM remodeling to growth plate angiogenesis at the growth plate.

## 24 WISP3 AND PROGRESSIVE PSEUDORHEUMATOID DYSPLASIA

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Loss of function mutations in WISP3 cause progressive pseudorheumatoid dysplasia (PPD), a human autosomal recessive disease characterized by precocious joint failure. We have identified putative disease causing mutations in 10 of 17 families that were referred to us with the clinical diagnosis of PPD. We have been able to obtain affected joint tissue, at the time of total joint replacement surgery, from two teenage patients with WISP3 mutations. Histological analysis in each patient revealed changes restricted to the articular cartilage surface and underlying subchondral bone, without involvement of meniscus or synovium. To explore the role of WISP3 in cartilage we have used *in situ* hybridization and northern blot analysis to identify sites of Wisp3 expression in mice and in a variety of human and mouse-derived cultured cell lines. To date, we have been unable to document Wisp3 expression in specific cells or tissues other than by reverse transcription polymerase chain reaction. We targeted the Wisp3 gene in mice, by creating a predicted loss of function allele. Surprisingly, these targeted mice have no obvious phenotype, even after being aged for over a year. We are currently trying to determine whether subtle skeletal phenotypes exist or can be induced by environmental factors. Finally, we made a polyclonal anti-WISP3 antibody that can recognize a shared human and mouse WISP3 epitope on western blots and have created cell lines that express WISP3. We are currently using these resources to evaluate the processing, post-translational modification, supramolecular assembly, and biological activity of this protein.

## 25 THE ROLES OF CTGF/HCS24, A HYPERTROPHIC CHONDROCYTE-SPECIFIC GENE PRODUCT 24, IN CARTILAGE, BONE AND VASCULAR FORMATION

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We previously reported that connective tissue growth factor/hypertrophic chondrocyte-specific gene product 24 (CTGF/Hcs24) stimulated proliferation, differentiation (maturation and hypertrophy) and calcification of cultured rabbit growth cartilage (RGC) cells and proliferation, differentiation and calcification of cultured osteoblastic cells. We also reported that CTGF/Hcs24 is a novel, potent angiogenesis factor in vitro and in vivo. From these findings, we proposed that in a physiological state, CTGF/Hcs24 produced by hypertrophic chondrocytes promotes endochondral ossification by acting on three types of cells as a paracrine factor, which may be called "ecogenin: endochondral ossification genetic factor". In this meeting, we will report on the role of CTGF/Hcs24 in cartilage, bone and vascular formation in more detail.

CTGF/Hcs24 bound to perlecan, a heparan sulfate proteoglycan, on the surface of chondrocytic HCS-2/8 cells. Heparinase treatment released CTGF/Hcs24 from HCS-2/8 cells into the culture medium, and reduced CTGF/Hcs24-stimulated aggrecan gene expression and DNA/proteoglycan synthesis in HCS-2/8 cells. These inhibitory effects were restored by the addition of heparan sulfate, indicating that the effects of CTGF/Hcs24 on chondrocytes occurred through the interaction between CTGF/Hcs24 and heparan sulfate on the cells. Immunostaining using rat growth plate revealed that in contrast to CTGF/Hcs24, heparan sulfate was localized in proliferative and premature zones but not in the hypertrophic zone. Consistent with such findings in vivo, the binding of <sup>125</sup>I-recombinant CTGF/Hcs24 to chondrocytes declined as the cells differentiate toward hypertrophy. These findings suggest that heparan sulfate proteoglycans, such as perlecan, play an important role in the paracrine action of CTGF/Hcs24 in growth plate cartilage during endochondral ossification.

CTGF/Hcs24 stimulated DNA/proteoglycan synthesis and gene expression of aggrecan and collagen type II, which are markers of chondrocyte maturation, in rabbit articular cartilage (RAC) cells. However, gene expression of tenascin-C, a marker of articular chondrocytes, was stimulated by CTGF/Hcs24 in RAC cells, but not in RGC cells. Moreover, CTGF/Hcs24 did not induce hypertrophy of articular chondrocytes or calcification of their matrix. When CTGF/Hcs24, in conjunction with gelatin hydrogel as a carrier, was injected into the knee joints of rats suffering from experimental osteoarthritis, articular cartilage defects were repaired. Moreover, implantation of CTGF/Hcs24 with gelatin hydrogel into a cartilage defect made by a drill also promoted repair of articular cartilage with no calcification, suggesting that it may be useful for the repair of articular cartilage.

Although gene expression of CTGF/Hcs24 in osteoblasts and osteocytes in vivo is much lower than that in hypertrophic chondrocytes, mechanical stress induced CTGF/Hcs24 expression in rat osteoblasts and osteocytes in vivo, indicating that CTGF/Hcs24 is involved in bone formation and remodeling. Implantation of CTGF/Hcs24 with gelatin hydrogel into calvaria defects made by a drill resulted in direct bone formation in the defects. Moreover, CTGF/Hcs24 was expressed in osteoblast-like cells and endothelial cells during the process of rat alveolar bone regeneration after tooth extraction. These findings indicate that CTGF/Hcs24 plays important roles in angiogenesis, bone formation or remodeling when stimulated or administered exogenously.

Previously, we showed that a high level of expression in hypertrophic chondrocytes was related to angiogenesis into cartilage from bone during the process of endochondral ossification. In addition to this physiological state, we found that highly vascularized tumors produced a large amount of CTGF/Hcs24 and that hypoxia induced gene expression and production of CTGF/Hcs24 in tumor cells, indicating that CTGF/Hcs24 is also a pathological angiogenesis factor.

CTGF/Hcs24 stimulated proliferation of cultured periodontal ligament (PDL) cells and the expression of several osteoblastic markers, such as alkaline phosphatase and collagen type I. However, the growth factor also stimulated gene expression of periostin, a marker of PDL and periosteum but not of osteoblasts, in the cells. When sparsely plated, the periodontal ligament cells expressed CTGF/Hcs24, suggesting a role for this molecule in the regeneration of PDL.

Taken together with our findings about gene expression of CTGF/Hcs24 during fracture healing and about skeletal disorders in CTGF/Hcs24 transgenic mice and the rescue of cartilage differentiation in cbfa1-null mice by the transgenic mice, the above findings indicate that although the major physiological role of CTGF/Hcs24 is to promote endochondral ossification as a paracrine factor, the growth factor is also involved in direct bone formation, angiogenesis in various tissues, including tumors, and PDL formation, when induced by certain stimuli. The stimulatory effects of exogenous administration also suggest future clinical applications for the regeneration of cartilage, bone and other skeletal tissues and the vascular system.

## 26 CHARACTERISATION OF CONNECTIVE TISSUE GROWTH FACTOR (CTGF) DURING XENOPUS DEVELOPMENT

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Connective tissue growth factor (CTGF) is a member of a newly identified family of extracellular matrix-associated signalling molecules known as the CCN family. CTGF is able to mediate cell adhesion, promote cell migration and enhance growth factor-induced cell proliferation in vitro, and to induce angiogenesis in vivo. It has also been implicated in wound healing and tumorigenesis. Recently, it has been shown that CTGF is a novel ligand of the cell surface receptors integrins.

Little is known about CTGF function in vivo or its role in development and loss of function mutants have not been reported to date. We have investigated the role of CTGF during development using *Xenopus laevis* as a model system. We first analysed the spatial and temporal expression of xenopus CTGF (xCTGF) during embryogenesis to identify the tissues and cell types in which it functions. xCTGF is first expressed in the early neurula, and is present until at least the early tadpole stage. Its transcripts are localised to a variety of tissues, as already shown for the mouse homologue, indicating that CTGF might have multiple roles during development.

To understand the role of xCTGF during embryogenesis, we injected xCTGF synthetic RNA into embryos and analysed the resulting phenotypes. The majority of embryos had a short trunk and abnormal heads, compared with controls. This overexpression phenotype indicates that xCTGF can interfere with anterior-posterior patterning of the embryo, since the body axis is shorter and anterior structures like the cement gland are expanded. Similar effects to xCTGF overexpression have been observed with overexpression of antagonists of the Wnt signalling pathway. This pathway has been implicated in anterior-posterior patterning and in the control of morphogenetic movements. We are now investigating the interaction of CTGF with the Wnt pathway. Preliminary data suggest that it can antagonise this pathway and we are currently testing if it can act by directly binding Wnts, their receptors Frizzled or the recently identified co-receptor, LRP6.

## 27 WISP-1 PROMOTES METASTASIS BY INDUCTION OF HYALURONAN AND CD44

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Abstract not available.

## 28 WISP-2 GENE IN HUMAN BREAST CANCER: ROLE OF ESTROGEN AND PROGESTERONE

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WISP-2 mRNA and protein were overexpressed in pre-neoplastic and cancerous cells of human breast. Statistical analyses show a significant association between WISP-2 expression and estrogen

receptor (ER) positivity. In normal breast, WISP-2 expression was virtually undetectable. The studies showed that WISP-2 is an estrogen-induced early response gene in MCF-7 cells and that expression continuously increased to reach a maximum level at 24 hours. The estrogen effect was inhibited by a pure antiestrogen (ICI 182,780). HMEC, in which WISP-2 expression was undetected or minimally detected, responded to 17 $\beta$ -estradiol by upregulating the WISP-2 gene after transfection with ER- $\alpha$ , providing further evidence that WISP-2 expression is mediated via ER- $\alpha$ . Overexpression of WISP-2 mRNA by estrogen may be accomplished by both transcriptional activation and stabilization. MCF-7 cells exposed to progesterone had a rapid but transient increase in WISP-2 expression. In combination with estradiol, progesterone acted as an antagonist, inhibiting the expression of WISP-2 mRNA. Moreover, disruption of WISP-2 signaling in MCF-7 cells by use of antisense oligomers caused a significant reduction in tumor cell proliferation. The results are consistent with the conclusion that WISP-2 expression is a requirement for breast tumor cell proliferation.

### 29 WISP3 IS A NOVEL TUMOR SUPPRESSOR GENE FOR INFLAMMATORY BREAST CANCER

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**Introduction:** Inflammatory breast cancer (IBC) is an aggressive and metastatic form of breast cancer with a 5-year disease-free survival of less than 45%. Little is known of the genetic alterations that result in IBC. Previous work from our laboratory found that WISP3 was specifically lost in IBC when compared with stage-matched, non-IBC. We hypothesize that WISP3 loss is a specific genetic alteration that determines the unique IBC phenotype.

**Methods:** We cloned and sequenced the full length WISP3 cDNA, and then cloned it into an expression vector. The resulting construct was introduced into the highly aggressive SUM149 IBC cell line that lacks WISP3 expression. We studied the morphological features of the transfectants, as well as the anchorage independent growth in soft agar, angiogenesis (enzyme linked immunosorbent assay method for vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), and functional rat aortic ring angiogenesis assay), and invasion and intravasation using the sea urchin assay. We investigated the effect of restoration of WISP3 expression on in vivo tumor growth in nude mice.

**Results:** The stable WISP3 transfectants had a round and vacuolated morphology compared with the controls. In soft agar assays, WISP3 transfectants formed 6–16 times less colonies than the transfection control cells ( $p < 0.05$ ). Stable WISP3 transfectants lost their ability to invade and intravasate. When evaluated for the production of angiogenic factors, the transfected cells produced significantly less VEGF and bFGF than the controls ( $p < 0.05$ ). The functional analysis of angiogenesis demonstrated that the transfected cell medium produced less new vessels from the preexisting aortic ring than the controls (26.33#61617; 1.63 v 69.33#61617; 4.15,  $p < 0.01$ ). Restoration of WISP3 expression resulted in decreased in vivo tumor growth in nude mice ( $p < 0.01$ ).

**Conclusions:** WISP3 may function as a tumor suppressor gene in the development of inflammatory breast cancer, and probably plays an important role in its growth and angiogenic properties.

### 30 DETERMINATION OF A POTENTIAL ROLE OF THE CCN FAMILY OF GROWTH REGULATORS ON GAP JUNCTIONS IN GLIOMA CELLS

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Tumour cells often exhibit erratic cell growth, as well as decreased gap junctional intercellular communication (GJIC). C6 glioma cells exhibit low levels of gap junction mRNA and protein, and decreased amounts of GJIC when compared with normal glial cells. We have previously shown that C6 glioma cells transfected with cDNA encoding the gap junction protein connexin43 (C6-Cx43) exhibit decreased proliferation in vivo and in vitro. To determine whether the expression of other genes is altered in response to an increased amount of Cx43 expression and intercellular coupling, C6 and C6-Cx43 cells were analysed for changes in gene expression by differential display.<sup>5</sup> One of the identified genes, Cyr61, was shown to be upregulated in C6-Cx43 cells, compared with C6 cells. Cyr61 is a member of the CCN gene family of growth regulators.<sup>6</sup> The expression of three other members has also been examined: WISP-1/Elm-1, WISP-2/Cop-2,

and WISP-3. These genes all share a highly conserved modular domain structure, and produce proteins that are rich in cysteine and secreted into the extracellular matrix. Various members of this family have been associated with many functions, such as migration, adhesion, and proliferation, as well as tumorigenesis and angiogenesis. Since Cyr61 has been shown to be upregulated in Cx43-expressing cells, our work aims at determining a potential function of the CCN genes in Cx43-transfected C6 glioma cells.

In this study, RNA levels of two CCN (connective tissue growth factor (CTGF), Cyr61/Cef-10, nephroblastoma overexpressed (NOV)) gene family members are shown to be upregulated in C6-Cx43 cells: Cyr61 and Nov. CYR61 has previously been shown to increase adhesion, migration, and growth in many cell types. Cyr61 RNA expression is shown here to respond to serum in quiescent cells in an immediate early gene fashion, independent of Cx43 expression. In comparison, NOV is shown to have growth suppressive capacities. Furthermore, confocal microscopy indicates that NOV colocalizes with Cx43 plaques at the cell membrane. These findings suggest interactions between CCN proteins and connexins, and provide insight into the possible role of Nov and Cyr61 in tumour cells.

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### 31 CONNECTIVE TISSUE GROWTH FACTOR IS EXPRESSED IN MALIGNANT ASTROCYTIC TUMORS AND IS INVOLVED IN CELL-CYCLE REGULATION

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Connective tissue growth factor (CTGF) is a member of an emerging family of extracellular matrix-associated heparin-binding growth factors, the CCN family, which have been implicated in tumor angiogenesis, cell adhesion and cell proliferation. There is increasing evidence that members of this family of growth factors mediate these signals through interactions with integrins, such as  $\alpha$ V- $\beta$ 3. Previous work has demonstrated that CTGF is upregulated in astrocytes in response to central nervous system injury. CTGF has also been shown to be expressed in glioma cell lines. The role of this growth factor in the pathogenesis of malignant gliomas has yet to be defined. We have performed an immunohistochemical analysis of CTGF expression in 29 human glial tumor specimens. Cytoplasmic localization of CTGF was found predominantly in tumor cells within high-grade tumors. CTGF expression was detected in 2 of 4 juvenile pilocytic astrocytomas, 0 of 6 grade II astrocytomas, 3 of 5 grade III astrocytomas, and 11 of 14 grade IV astrocytomas. Expression of CTGF within the tumor vasculature was limited to the juvenile pilocytic astrocytoma specimens. These results suggest that CTGF expression by astrocytoma cells may correlate with grade in adult gliomas and therefore may have prognostic significance.

To test the hypothesis that CTGF is involved in the proliferation of glioma cells, we treated glioma cell lines that constitutively produce this cytokine with morpholino antisense oligonucleotides designed to inhibit the translation of CTGF. Treatment with antisense CTGF was associated with a marked reduction in CTGF, as demonstrated by immunoblot analysis of the conditioned medium of these cells compared with cells treated with a nonsense or missense morpholino. Antisense CTGF had no effect on levels of basic fibroblast growth factor or vascular endothelial growth factor. Cell cycle analysis revealed that downregulation of CTGF protein expression in the conditioned medium was associated with at least a 40% increase in the number of cells arrested at the G1/S transition of the cell cycle. Overexpression of CTGF by retroviral transduction of G55 cells resulted in a decrease in the number of cells in G1, with a near two-fold increase in the number of cells in the S-phase of the cell cycle, compared with the vector control.

We have begun to explore the mechanistic basis for the CTGF-mediated proliferative response and have shown by gene expression profiling of approximately 40 000 genes that downregulation of CTGF with antisense treatment is associated with changes in the expression of at least 50 genes, several of which potentially have a role in proliferation. Greater than twofold differences have been detected in the levels of expression of cyclin D1, cyclin G2, tumor necrosis factor receptor, DR-1 associated transcriptional corepressor, cadherin3, and fibroblast growth factor receptor 1.

We have begun to explore the physiological regulation of CTGF expression in glioma cells and have shown that the level of expression of CTGF in conditioned medium was markedly increased by the treatment of cells with a pharmacological antagonist of the phosphoinositid-3 (PI-3) kinase pathway. These results were confirmed by TAQMAN quantitative reverse transcription polymerase chain reaction, which revealed a near fourfold increase in CTGF expression with PI-3 kinase inhibition. Under these conditions, we demonstrated that while CTGF levels increase, inhibition of PI-3 kinase resulted in a marked decrease in the level of expression of vascular endothelial growth factor, an important mediator of brain tumor angiogenesis.

These results suggest that CTGF may constitute a pathway of autocrine regulation of astrocytoma growth and survival in the response to injury or nutrient deprivation.

### 32 CONNECTIVE TISSUE GROWTH FACTOR INCREASED BY HYPOXIA MAY INITIATE ANGIOGENESIS IN COLLABORATION WITH MATRIX METALLOPROTEINASES

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Connective tissue growth factor (CTGF) is known to be a potent angiogenic factor. Here, we investigated how CTGF and matrix metalloproteinases (MMPs) are involved in the early stage of hypoxia-induced angiogenesis using human breast cancer cell line, MDA231, and vascular endothelial cells. Hypoxic stimulation (5% O<sub>2</sub>) of MDA231 cells increased their steady-state level of ctgf mRNA approximately twofold within 1.5 hours, and the levels remained at a plateau up to 6 hours, and then decreased by 12 hours, as compared with the cells cultured under normoxic conditions. Membrane-type MMP mRNA levels were also increased within a few hours of exposure to hypoxia. Indeed, enzyme linked immunosorbent assay revealed that the amount of CTGF protein/cell in medium conditioned by MDA231 cells exposed to hypoxia was maximally greater at 24 hours than in the medium from normoxic cultures and that the secretion rate (supernatant CTGF/cell layer CTGF) increased in a time-dependent manner from 24 to 72 hours after hypoxic exposure. Hypoxic induction of CTGF was also confirmed by immunohistochemical analyses. Furthermore, zymogram analysis revealed that the production of active MMP-9 was also induced in MDA231 cells incubated under hypoxic conditions. Finally, we found that recombinant CTGF also increased the expression of several metalloproteinases that play a role in vascular invasive processes and decreased the expression of tissue inhibitors of metalloproteinases (TIMPs) by vascular endothelial cells. These findings suggest that hypoxia stimulates MDA231 cells to release CTGF as an angiogenic modulator, which initiates the invasive angiogenesis cascade by modulating the balance of extracellular matrix synthesis and degradation via MMPs secreted by endothelial cells in response to CTGF. This cascade may play critical roles in the hypoxia-induced neovascularization that accompanies tumor invasion in vivo.

### 33 DEREGULATION OF NOV EXPRESSION IN CHRONIC MYELOID LEUKAEMIA

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Chronic myeloid leukaemia (CML) is characterized by the presence of the chimaeric bcr-abl fusion gene encoding a constitutively active protein tyrosine kinase. We have expressed a temperature sensitive mutant of the Bcr-Abl protein in a murine multipotent haemopoietic stem cell line, FDCP-Mix, and applied DNA microarray analysis to investigate the initial effects of this kinase on gene expression.

RNA was prepared from cells expressing the Bcr-Abl tyrosine kinase and from control cells at 3, 6, 12 and 24 hours. RNA was pooled, labelled with biotin and hybridised to an Affymetrix Mu6500 array (Affymetrix® USA). The Bcr-Abl kinase activity resulted in differential expression of 300 genes. Several expression signatures known to be associated with CML were identified. The array also detected a 25-fold decrease in the expression of a novel gene nephroblastoma overexpressed, or Nov, as a consequence of activating Bcr-Abl.

Northern blot analysis was used to confirm the differential expression and detected a 4-fold reduction of Nov mRNA in cells expressing Bcr-Abl through each of the 3–24 hour timepoints. Investigation of Nov protein by western blot analysis identified a 2-fold change in expression in the FDCP-Mix cells as a result of the Bcr-Abl tyrosine kinase. Confocal microscopy also revealed a significant decrease in intensity of Nov expression as a consequence of Bcr-Abl activity. FDCP-Mix control cells uniformly exhibited a high level of Nov fluorescence, whereas fluorescence was barely detectable in cells expressing Bcr-Abl. A similar pattern of Nov expression was observed in primary human cells (4 CML, 4 normal controls). Normal progenitor cells uniformly expressed an intense fluorescence signal for Nov expression whereas, in contrast, most of the cells within the CML patient samples were negative. A small subpopulation of cells within the CML patient samples were weakly positive for Nov fluorescence, possibly representing a residual population of normal cells. Previously, Nov was found to regulate cell growth and adhesion in vascular smooth muscle cells and a variety of tumour cell lines.

DNA microarrays provide a powerful tool for monitoring changes in gene expression. Using this technology, we have identified a novel gene Nov and a series of molecules that interact with Nov that may comprise a molecular pathway dysregulated in CML.

### 34 BIOLOGICAL ROLE OF NOVH IN EWING'S SARCOMA CELLS

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NovH protein is generally expressed in the cells of the musculoskeletal system, indicating that this protein may play a role both in normal and pathological conditions. However, the regulation of NovH expression varies in different neoplasms and depends upon the type of cells. An association between NOVH expression and tumour differentiation was observed in rhabdomyosarcoma and cartilage tumours, whereas, in Ewing's sarcoma, the expression of NovH was found to be associated with a higher risk of developing metastases in clinical samples. To clarify the role of this molecule in the biology of Ewing's sarcoma cells, we have transfected the TC-71 Ewing's sarcoma cell line with plasmids containing NOVH sequences under different promoters (inducible or not) to obtain stable clones expressing high levels of the protein. The in vitro behaviour of these clones was analysed with respect to their growth and migratory ability. All four TC/NOVH transfectants analysed showed significantly lower growth ability both in monolayer and soft-agar conditions. The doubling time of these clones was significantly higher, probably because of a remarkable increase in the apoptotic rate of the TC/NOVH clones under basal conditions. These data appeared to conflict with previous findings in clinical samples. However, when we analysed the migratory ability of NOVH expressing transfectants, a significant increase was found compared with the migration of parental cells. Similar results were obtained with clones expressing NOVH in inducible conditions. These findings therefore indicate a controversial role of NOVH protein in Ewing's sarcoma cells. A better analysis of the molecular intracellular mechanisms responsible for mitogenesis and migration of Ewing's sarcoma cells with respect to the expression of NOVH is in progress.

### 35 CCN5 INHIBITS THE PROLIFERATION AND MOTILITY OF HUMAN MYOMETRIAL AND LEIOMYOMA SMOOTH MUSCLE CELLS

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Uterine fibroids are a significant problem in women's health. The only treatment option currently available with minimal recurrence risk and reduction of symptoms is hysterectomy. Because leiomyomas are benign tumors of smooth muscle cells, one approach to developing therapeutic rationales is to elucidate the mechanisms that regulate uterine smooth muscle cell proliferation and to search for molecules that can inhibit uterine smooth muscle cell mitogenesis. Two candidate molecules are the glycosaminoglycan heparin and CCN5, a heparin-induced gene and member of the CCN family. We have previously shown that heparin is capable of inhibiting both vascular and uterine smooth muscle cell proliferation and motility. In addition, we have demonstrated that overexpression of CCN5 using an adenoviral construct inhibits vascular smooth muscle cell proliferation and motility,

but does not affect adhesion and apoptosis. Here, we demonstrate that the proliferation and motility of both human myometrial and leiomyoma smooth muscle cells are inhibited by overexpression of CCN5. When compared with cells infected with green fluorescent protein control adenovirus (AdGFP), the proliferation of CCN5-infected cells (AdCCN) is inhibited by up to 60%, depending on the multiplicity of infection (MOI). Using a scratch wound assay, our results indicate that AdCCN5 at MOI as low as 10 results in a 33% reduction in migration compared with AdGFP-infected cells. Experiments are currently in progress to determine whether CCN5 influences cell death in uterine smooth muscle cells. We are also examining the role of heparin in the induction of CCN5 expression and whether myometrial and leiomyoma smooth muscle cells express similar levels of CCN5.

### 36 CYR61 IS ASSOCIATED WITH ENDOMETRIOSIS

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Following retrograde menstruation, shed endometrial fragments attach to and invade the peritoneal surface and establish endometriotic lesions. An inappropriate differentiated endometrium caused by anomalous expression of genes may be the reason for the development of these endometriotic lesions. DNA microarray analysis was used to identify genes that are differentially expressed in endometria from women with endometriosis compared with endometria from women without endometriosis. Of the several genes found to be differentially expressed, Cyr61, a member of the CCN family of growth and angiogenic regulators, was shown to be upregulated in the endometrium of women with endometriosis in 90% of cases (n = 20). Real-time reverse transcription polymerase chain reaction (RT-PCR) experiments with TaqMan technology using SYBR green dye corroborate the results of the microarray analysis for Cyr61 using additional samples of endometrial tissue. In further experiments, upregulation of Cyr61 in endometriotic lesions was measured by routine RT-PCR. Independent of the cyclic phase, higher expression levels of Cyr61 could be detected in endometriotic lesions than in the corresponding endometria. Cyr61 is located in gland epithelium of the endometria as well as in endometriotic lesions, but not in stromal cells. To examine regulators of Cyr61 expression we used an established nude mouse model<sup>1</sup> and transplanted human endometrial fragments into the peritoneum of nude mice. The animals were treated with 17 $\beta$ -estradiol, anti-estrogen or vehicle over a time period of 14 days. It could be shown that Cyr61 is regulated directly by estrogen—in mice treated with anti-estrogen the expression of mRNA and protein is downregulated. Thus, Cyr61 is a marker gene for endometriosis and may play a role in the development of the disease.

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### 37 CCN2 (CTGF) IN DIABETES: A PREDICTOR OF PROGRESSIVE KIDNEY DISEASE

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There is currently a great need to be able to predict which newly diagnosed diabetic patients are among the 30–40% that will develop nephropathy and progress to kidney failure. Microalbuminuria, currently thought by some to predict nephropathy, actually determines established glomerular involvement. We have shown that CCN2 (CTGF) is greatly upregulated in the glomerulus early in experimental diabetes, and kidney mesangial cells exposed to recombinant human CCN2 are stimulated to increase production of those extracellular matrix molecules responsible for glomerulosclerosis.<sup>8</sup> Furthermore, in a small study of diabetic patients, we found that glomerular CTGF mRNA levels in albuminuric patients were not unlike those with overt proteinuria, but were significantly higher than in living related donors.<sup>9</sup> We therefore investigated whether urinary CCN2 might serve to predict and stage progression of renal disease. Low levels of urinary CCN2 were present in healthy rats, but were increased approximately 7-fold, overall, in STZ-induced diabetic rats. Levels were highest early, at week 3 of diabetes, then decreased with time, but remained significantly increased over controls even after 32 weeks. Likewise, consistently low levels of urinary CCN2 were detected in healthy volunteers (mean value, 7.1 CCN2/mg creatinine). However, levels were greatly raised in 70% of diabetic patients with nephropathy, whereas 30% of the diabetic patients not

yet exhibiting evidence of renal involvement had increased levels of urinary CCN2. To examine the relationship between CCN2 levels and the stage of progression, we studied 18 patients with proteinuria and/or raised serum creatinine. Blood pressures were also measured. CCN2 levels increased as kidney function decreased, as determined by increased proteinuria ( $R_s$ , 0.322;  $p < 0.0001$ ) and serum creatinine levels ( $R_s$ , 0.21;  $p < 0.0003$ ). Furthermore, there was a highly significant correlation between CCN2 levels and mean arterial blood pressures ( $R_s$ , 0.878;  $p < 0.0001$ ). No significant differences were detected between groups receiving angiotensin-converting enzyme or HMG-CoA synthetase inhibitors, or angiotensin receptor blockers. These data support our hypothesis that CCN2 is upregulated early in the evolution of renal fibrosis/glomerulosclerosis, including that of diabetes. We contend that the measurement of urinary CCN2 may provide a rapid non-invasive method to stage nephropathy and to predict those patients destined for progressive sclerosis and end stage disease.

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### 38 EARLY UPREGULATION OF CONNECTIVE TISSUE GROWTH FACTOR (CTGF) PROTEIN LEVELS IN PODOCYTES AND URINE OF STREPTOZOTOCIN-INDUCED DIABETIC MICE

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Overexpression of mRNA encoding the profibrotic growth factor CTGF has been reported in diabetic glomeruli. We studied renal and urinary CTGF protein expression in the early phase of insulin-dependent diabetes mellitus (IDDM) in C57Bl/6J mice.

Diabetic and age-matched control mice were sacrificed 2, 3, 4 and 8 weeks after IDDM induction (STZ, single injection; 200 mg/kg intraperitoneally). Renal CTGF protein expression was determined by immunohistochemistry. CTGF in urine was determined by sandwich enzyme linked immunoassay.

Histologically, diabetic mouse kidneys showed a 16% increase of glomerular tuft area compared with controls. Morphometry of CTGF staining revealed an 8-fold increase of CTGF protein expression in the glomeruli of diabetic mice compared with controls. CTGF staining was mainly confined to podocytes as demonstrated by colocalization with WT-1 staining. Diabetic mice were proteinuric and urinary CTGF levels ranged from 20 to 50 ng/ml, while CTGF was < 5 ng/ml in the urine of control mice. If corrected for creatinine, this amounts to at least a 10-fold increase in levels of urinary CTGF (ng CTGF/mg creatinine) in diabetic versus control mice.

Thus, CTGF protein is upregulated in podocytes of diabetic mice very early after the onset of hyperglycemia. An increase of CTGF expression is also demonstrable in urine samples of diabetic mice. In the light of previous in vitro reports emphasizing glucose-induced overexpression of CTGF by mesangial cells, the predominant expression in podocytes is unexpected, although similar to that reported for VEGF expression in diabetic glomeruli.

We suggest that CTGF may be a valuable marker and/or pathogenic factor in the development of diabetic nephropathy.

### 39 PLASMA LEVEL OF CONNECTIVE TISSUE GROWTH FACTOR (CTGF) IN DIABETES MELLITUS CORRELATES WITH ALBUMINURIA

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CTGF is a 36–38 kDa secreted protein, which is greatly upregulated in fibrotic disorders, including diabetic nephropathy (DN). In this study we investigated the relationship of the plasma CTGF level with markers of nephropathy, metabolic control, platelet activation, and

endothelial dysfunction in patients with complicated and uncomplicated diabetes mellitus (DM). CTGF levels were determined by enzyme linked immunosorbent assay in citrate plasma samples from a cross sectional cohort of 21 healthy control subjects and 64 patients with type I DM with or without microalbuminuria (MA; 30–300 mg/day) and overt DN (> 300 mg/day). A Kruskal-Wallis analysis followed by Dunn's method was used to determine differences in plasma CTGF levels between control subjects and patient groups. Forward stepwise regression analysis was used to compare plasma CTGF levels with general patient characteristics (age, body mass index, blood pressure, duration), metabolic control (HbA1c), platelet activation (platelet count, von Willebrandt factor), endothelial dysfunction (vascular endothelial growth factor) and nephropathy (albuminuria, creatinine clearance). A p value < 0.05 was considered significant. DN patients showed significantly increased plasma CTGF levels compared with both control subjects and DM. Forward stepwise regression analysis in all patients showed that albuminuria and creatinine clearance were independent predictors of plasma CTGF levels. Moreover, within the subgroup of DN patients, plasma CTGF levels strongly correlated with albuminuria ( $r = 0.87$ ;  $p < 0.001$ ), but not with creatinine clearance.

We suggest that CTGF might be a valuable marker and/or pathogenic factor in the development of DN.

#### 40 CTGF, DISEASE AND THERAPEUTIC ANTIBODIES

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We have generated a collection of human monoclonal antibodies (mAbs) to CTGF and characterized these for binding affinity, specificity and bioactivity. One of these mAbs has been extensively studied and it can significantly reduce collagen deposition in rodent models of lung and kidney fibrosis.

For a better understanding of the role of CTGF in disease, we have examined the in vivo effects of recombinant human (rh) CTGF in novel animal model systems. A fibrosis model in neonatal mice has been developed to test the activity of rhCTGF and rhCTGF fragments. This model, which involves systemic exposure of transforming growth factor  $\beta$  (TGF- $\beta$ ) and CTGF, results in organ fibrosis and death. These effects are rhCTGF dose-dependent and prevented by treatment with a CTGF-specific mAb. In another animal model, rhCTGF was shown to enhance angiogenesis.

To establish a link between CTGF action in these animal models and human fibrotic disease, we have determined CTGF content in a wide variety of patient samples. Three sandwich enzyme linked immunosorbent assays to quantitate full length CTGF, N-terminal and C-terminal CTGF halves were developed using pairs of mAbs. Immunohistochemical methods were also developed to examine the tissue localization of CTGF. Findings from these studies demonstrate that: (1) CTGF content is increased locally in numerous fibrotic conditions; (2) CTGF content is also increased systemically (in plasma and/or urine) in fibrosis patients; (3) plasma and urine levels of CTGF correlate with disease severity; and (4) the CTGF N-fragment is a good indicator of fibrosis.

These results demonstrate a central role for CTGF in fibrosis and angiogenesis. They further suggest that distinct mechanisms may allow blockade of CTGF action and that CTGF inhibitors are likely to be beneficial therapies for disease conditions involving fibrosis and angiogenesis.

#### 41 NOV IS ASSOCIATED WITH CX43 BUT NOT WITH CX40 AND TRCX43 IN HUMAN CHORIOCARCINOMA TRANSFECTANTS: EVIDENCE FOR POTENTIAL GROWTH-REGULATORY FUNCTIONS

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Gap junctions are clusters of intercellular channels composed of connexin proteins between adjacent cells, which mediate direct cell–cell communication. To date, 20 different connexins in the human genome are known, which build up channels of different physiological properties. The differences between the connexins are mainly due to the C-terminal region, which plays an important role in signal transduction processes. We have focused on the functional implication of connexins in trophoblast cells of the developing placenta. Cx40 is detected within the cytotrophoblast of human placenta, and is restricted to the proximal extravillous trophoblast cells of cell columns in first trimester placentas, which are highly proliferative.<sup>10</sup> Cx43 has been implicated in the differentiation of villous stem cytotrophoblasts towards syncytiotrophoblast by cell fusion and in the invasive extravillous trophoblast differentiation into giant cells.<sup>11</sup> The human malignant trophoblast cell line Jeg3, which express only small amounts of Cx40 mRNA, represents a model for the proliferative trophoblast. Jeg3 cells grown in nude mice eroded host vessels and replaced the endothelial lining in a similar way to trophoblast cells during the establishment of the placenta.<sup>12</sup> For this reason, this model is most suitable to investigate the role of different connexins for trophoblast cell proliferation, differentiation and invasion.

In this study, we established stable Jeg3 Cx40, Cx43 and truncated Cx43 (trCx43; Cx43 without C-terminus) transfectants under the control of a tet-on system. After characterization of the clones at the mRNA, protein and functional levels we evaluated in vitro cell growth and in vivo growth characteristics of the different Cx transfectants in nude mice. Restoration of cell–cell communication via Cx43 protein channels reduced significantly cell growth in vitro and tumor growth in vivo. In contrast, Jeg3 Cx40 and trCx43 transfectants revealed no obvious changes in cell proliferation in vitro as well as in tumor growth in vivo. Gene array analysis of the different Cx transfectants and validation by the reverse transcription polymerase chain reaction revealed an upregulation of NOV, a member of the CCN family with negative growth-regulatory functions, in the Jeg3 Cx43 transfectants. Double immunolabeling of NOV and Cx43 in induced Cx43 transfectants demonstrated a colocalization of NOV and Cx43 at the cell membrane. In contrast, in parental Jeg3 cells and in Cx40 as well as in trCx43 transfectants NOV could be detected predominantly in the nucleus. The association of NOV and Cx43 at the cell membrane could be responsible for the growth reduction of the Jeg3 Cx43 transfectants.

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#### MEETING DETAILS AND ACKNOWLEDGEMENTS

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