CCN abstracts

Oral communications

O1 CTGF EXPRESSION IS SENSITIVE TO CHANGES OF THE ACTIN CYTOSKELETON IN ENDOTHELIAL CELLS

S. Muehlich[1], B. Krueger[1], C. D. Garlichs[2], M. Goppelt-Struebe[1]. [1]*Medical Clinic IV and* [2]*Medical Clinic II, University of Erlangen-Nuernberg, Erlangen, Germany*

CTGF has been shown to be upregulated in human umbilical vein endothelial cells (HUVEC) under turbulent flow conditions. This prompted us to investigate the molecular mechanisms that might link changes in cell morphology and CTGF gene expression.

CTGF expression was studied in primary cultures of HUVEC. Manipulation of the actin cytoskeleton revealed a reverse correlation between CTGF expression and the cellular concentration of monomeric G-actin: when the actin cytoskeleton was disrupted by latrunculin, CTGF mRNA and protein synthesis decreased. When monomeric G-actin was reduced by enhanced formation of F-actin stress fibres by yasplakinolide, CTGF synthesis was increased. Similarly, disruption of microtubuli by colchicine and the subsequent increase in F-actin strongly enhanced CTGF synthesis.

To provide direct evidence for the role of G-actin in CTGF expression, endothelial cells were transfected with a construct encoding mutated G-actin, which was no longer able to polymerise into F-actin. This construct was kindly provided by G Posern and R Treisman (London, UK). Compared with mock transfected cells, actin transfected cells showed a reduced number of stress fibres. Endothelial cells are poorly transfectable cells. Therefore, the effect of the mutated G-actin on CTGF expression was assessed in individual cells by immunocytochemistry. CTGF was located to Golgi stacks. Comparison of transfected cells with neighbouring non-transfected cells showed a strong reduction of CTGF in transfected cells, which was not seen when the cells were transfected with an irrelevant vector. The molecular mechanism by which G-actin interferes with CTGF synthesis remains to be elucidated.

Similar data were obtained in a murine microvascular endothelial cell line and also in fibroblasts, indicating that independent of the cell type, CTGF expression seems to be sensitive to changes in cell morphology.

O2 CCN2 GENE REGULATION IN CHONDROCYTIC DIFFERENTIATION

S. Kubota[1], Y. Mukudai[2], T. Eguchi[1], S. Kondo[1], M. Minato[1], H. Mimura[1], M. Takigawa[1,2]. [1]*Department of Biochemistry and Molecular Dentistry, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan;* [2]*Biodentistry Research Centre, Okayama Dental School, Okayama, Japan*

The CCN2 gene is differentially expressed and plays central roles in the growth and differentiation of chondrocytes. The expression of ccn2 is highest in the prehypertrophic–hypertrophic layers, whereas it is low in the layers beneath the proliferative zones. To investigate the mechanism behind this regulation, we used chick primary cells and human chondrocytic HCS-2/8 cells.

First, comparative analysis using primary chick embryonic fibroblasts, lower sterna (representing proliferative zone) chondrocytes, and upper sterna (representing prehypertrophic–hypertrophic zones) chondrocytes revealed the involvement of both transcriptional and post-transcriptional mechanisms for the enhanced ccn2 gene expression during chondrocytic differentiation. Namely, transcriptional activity was enhanced as differentiation advanced; furthermore, ccn2 mRNA stability was also increased, which resulted in a net increase of the ccn2 steady state mRNA level during differentiation. Using reporter gene assays, the region on the ccn2 mRNA responsible for the post-transcriptional regulation localised to the 3′-untranslated region (UTR) of chick ccn2. Interestingly, the location of the regulatory element in the 3′-UTR was completely different from that of the cis acting element of structure anchored repression (CAESAR) in human and murine ccn2 3′-UTRs. Further investigations to clarify the post-transcriptional regulatory mechanism are currently under way.

The glucocorticoids are also known to be involved in regulation of the phenotypes of proliferative and mature chondrocytes. Here, we also found that dexamethasone strongly induced ccn2 gene expression and protein production from HCS-2/8 chondrocytic cells, which resulted in enhanced proteoglycan synthesis by those cells. Although the genetic element that is crucial for mediating this effect has not been clarified, it is certain that the glucocorticoid induction of ccn2 expression occurs at the transcriptional level. Efforts are being made to map the genetic element.

O3 REGULATION OF TGFβ1 and CTGF, in a model of smooth muscle cells isolated from radiation enteritis, by pharmacological inhibition of Rho–ROCK pathway

V. Haydont[1,2], D. Mathé[1], M. Benderitter[2], J. Bourhis[1], M. C. Vozenin-Brotons[1,2]. [1]*UPRES EA 27-10, Radiosensibilité des Tumeurs et Tissus Sains, IGR/IRSN, Villejuif, France;* [2]*Laboratoire d'étude des Pathologies Radio-induites, SRBE/DRPH, IRSN, Fontenay-aux-Roses, France;* [3]*Département de Radiothérapie, IGR, Villejuif, France*

Radiation enteritis is a pathological consequence of cancer treatment by radiotherapy, and is characterised by transmural fibrosis. Because connective tissue growth factor (CTGF) is known to be involved in the fibrogenic process we investigated its regulatory pathway in radiation enteritis. Several molecular pathways are known to control CTGF expression, including the Smad pathway activated by transforming growth factor β1 (TGFβ1)[1] and the Rho–ROCK pathway.[2] In this study, we investigated the relation between TGFβ1, CTGF, and the Rho–ROCK pathway using two pharmacological inhibitors: (1) Pravastatin, an inhibitor of HMG CoA reductase, which inhibits Rho isoprenylation; and (2) Y-27632, an inhibitor of ROCK activity.

In vitro culture models of primary smooth muscle cells were established from healthy bowel or radiation enteritis. Smooth muscle cells isolated from radiation enteritis showed high CTGF expression and a Rho/ROCK alteration compared with their normal counterparts. Cells were incubated with Pravastatin (100μM, 500μM, or 1000μM for three, six, or 24 hours), or with Y-27632 (50μM or 100μM for one, three, six, or 24 hours). Real time polymerase chain reaction (PCR) was used to study CTGF, TGFβ1, and collagen Iα2 mRNA values. CTGF protein expression was studied by western blotting.

Both inhibitors—Y-27632 and Pravastatin—induced a decrease of CTGF protein and mRNA values as soon as three hours after exposure. Surprisingly, TGFβ1 and collagen Iα2 mRNA values increased after three and six hours, whereas TGFβ1 mRNA decreased after 24 hours of incubation.

These observations confirm that the Rho–ROCK pathway controls CTGF expression in cells isolated from radiation enteritis. Moreover, the uncoupled expression of TGFβ1 and CTGF at an early time point suggests that TGFβ1 does not upregulate CTGF expression when the Rho–ROCK pathway is inhibited.

Acknowledgments: the study was supported by ''Le Comité de Radioprotection d'Electricité De France''. The authors wish to thank Dr S Quentzel (Bristol-Myers Squibb) for providing Pravastatin.

1. **Blom IE**, *et al. Matrix Biol* 2002;**21**:473–82.
2. **Eberlein M**, *et al. Br J Pharmacol* 2001;**133**:1172–80.

O4 INCREASED CCN2 TRANSCRIPTION IN KELOID FIBROBLASTS REQUIRES COOPERATIVITY BETWEEN AP-1 AND SMAD BINDING SITES

W. Xia[1,2], W. Kong[1], T-T. Phan[3], I. J. Lim[3], M. T. Longaker[1], G. P. Yang[1,4]. [1]*Department of Surgery, Stanford University Medical School, Stanford, California, USA;* [2]*Department of Plastic Surgery, Fourth Military Medical University, Xi'an, China;* [3]*Department of Surgery, National University of Singapore, Singapore;* [4]*Palo Alto VA Health Care System, Palo Alto, California, USA*

Keloids are proliferative dermal growths representing a pathological wound healing response. We used serum stimulation as an in vitro model to mimic a component of the wound microenvironment to examine differential gene expression in keloid derived fibroblasts versus normal human fibroblasts. We found that transcription of connective tissue growth factor (CTGF, CCN2) under basal conditions and after serum stimulation was significantly higher in keloid fibroblasts compared with normal fibroblasts. The transcriptional response was in an immediate early fashion, as described previously. Promoter analysis demonstrated that the fragment from −140 to −72 conferred increased basal expression and the fragment from −625 to −140 conferred

increased serum responsiveness. Mutational analysis showed that an AP-1 and Smad binding site were both necessary for serum responsiveness. The addition of antibodies to c-Jun, Smad2, or Smad3 was able to supershift the binding complex using either the AP-1 or Smad binding site oligonucleotide, indicating that transcriptional activation of CCN2 after serum stimulation is directed by a complex that interacts with both enhancer elements. The use of small molecule inhibitors of activation of either JNK or the transforming growth factor β receptor 1 blocked transcriptional activation of CCN2, whereas inhibitors of other signal transduction pathways did not. Additional studies showed that there are no differences in protein concentrations of these transcriptional activators in keloid versus normal fibroblasts. Smad3 translocates into the nucleus after serum stimulation in keloid and normal fibroblasts. We did detect significant differences in the activation of c-Jun, with far higher levels of c-Jun activation as measured by immunoblotting for phosphorylated c-Jun.

These data suggest that the mechanism of keloid pathogenesis may involve in part an inherent difference in how the fibroblasts respond to wounding. Specifically, CCN2 activation by serum stimulation is dependent upon both AP-1 and Smad signalling pathways, and significant differences were seen in AP-1 activation, suggesting that this is the crucial pathway that leads to enhanced CCN2 transcription in keloid derived fibroblasts.

O5 DIFFERENTIAL REGULATION OF CTGF/CCN2 TRANSCRIPTION IN MDA231 BREAST CANCER CELL LINE AND HCS-2/8 CHONDROCYTIC CELL LINE

T. Eguchi[1,3], S. Kubota[1], K. Kazumi[1], T. Ogawara[1], Y. Mukudai[2], M. Takigawa[1,2]. *[1]Department of Biochemistry and Molecular Dentistry, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan; [2]Biodental Research Centre, Okayama University Dental School, Okayama, Japan; [3]Japanese Society for Promotion of Science, Tokyo, Japan*

Connective tissue growth factor (CTGF/CCN2) is highly expressed in chondrocytes and other types of cells, where it regulates endochondral bone formation and tissue regeneration; in addition, it is expressed in breast cancer cells involved in metastasis to bone. Therefore, it is important to clarify the regulatory mechanism of ctgf/ccn2 gene expression with regard to these biological phenomena. To investigate cell type specific or tissue specific transcriptional regulation of the ctgf/ccn2 gene, we transfected an 800 bp human ctgf/ccn2 promoter–reporter construct and its mutants into the following human cell lines—the HCS-2/8 chondrocytic cell line, the MDA231 breast cancer cell line, the SaOS-2 osteoblastic cell line, and the HeLa cervical cancer cell line—and then carried out luciferase assays. We found that the relative promoter activities of ctgf/ccn2 were higher in HCS-2/8 and MDA231 and lower in SaOS-2 and HeLa, reflecting the relative mRNA and protein values among the cells tested. Mutagenesis in the Smad binding element or the transforming growth factor β (TGFβ) response element caused a pronounced reduction of promoter activity in MDA231, whereas it caused less of a reduction in HCS-2/8. Another cis element, TRENDIC (transcription enhancer dominant in chondrocytes), produced the highest enhancing activity in HCS-2/8 among the cells tested. These results suggest that ctgf/ccn2 expression is mainly activated through TGFβ/Smad signals in MDA231, whereas it is activated through TRENDIC and other cis elements in HCS-2/8.

O6 ENDOTHELIN-1 INDUCES CTGF THROUGH MEK/ERK AND BCE-1

X. Shi-wen, A. Holmes, C. M. Black, D. J. Abraham, A. Leask. *Centre for Rheumatology, University College London (Royal Free Campus), London, UK*

Endothelin-1 (ET-1) is upregulated during tissue repair and in fibrotic disease, including pulmonary fibrosis. Here, we show that the addition of ET-1 (100nM for four hours) to normal lung fibroblasts induces CTGF protein expression and extracellular signal regulated kinase (ERK) phosphorylation. Blockade of the MEK/ERK mitogen activated protein kinase pathway with U0126 prevents ET induced CTGF protein and mRNA expression. Promoter deletion and mutation analysis shows that ET-1 induces CTGF promoter activity through the previously identified basal control element-1 (BCE-1) site, but not through the transforming growth factor β (TGFβ)/Smad response element. We have shown that the overexpression of CTGF characteristic of fibroblasts taken from the fibrotic lesions of the disease scleroderma is independent of Smads but dependent on BCE-1. Our results suggest the possibility that endogenous ET-1 signalling may contribute to the overexpression of CTGF seen in scleroderma lesions. In addition, our results suggest that ET-1 and TGFβ cooperate to induce CTGF protein expression during wound healing.

O7 TISSUE SPECIFIC REGULATION OF CTGF BY PGE_2 AND ANALYSES OF SIGNAL TRANSDUCTION PATHWAYS IN HUMAN GINGIVAL FIBROBLASTS

S. A. Black Jr, M. Stan, A. H. Palamakumbura, N. Pischon, E. Heng, H. Yi, P. C. Trackman. *Department of Periodontology and Oral Biology, Boston University Goldman School of Dental Medicine, Boston, MA 02118, USA*

CTGF levels are raised in human gingival overgrowth tissues, particularly in patients treated with the anti-seizure drug phenytoin, and we suspect that CTGF promotes gingival fibrosis.

Gingival tissues always show some degree of inflammation. In addition, proinflammatory mediators including prostaglandin E2 (PGE_2) block transforming growth factor β (TGFβ) stimulated CTGF expression in cultured cell lines including IMR-90 and NRK cells. These findings suggest that PGE_2 does not downregulate CTGF in human gingival tissues and cells. Tissue specific differences in CTGF regulation could provide insight into the aetiology and tissue specificity of the pathology associated with drug induced gingival overgrowth. New studies presented here investigate the effects of PGE_2 on TGFβ1 stimulated CTGF mRNA levels, and investigate signalling pathways in human gingival fibroblast cultures, and in control human IMR-90 cells. Early passage gingival fibroblasts obtained from normal human explant cultures were grown under standard conditions to near confluence. Cells were serum starved and then pretreated with or without different concentrations of PGE_2 for one hour, followed by 5 ng/ml treatment with TGFβ1 (or no TGFβ1 control) for four hours. Total RNA was isolated and CTGF mRNA levels were then measured by real time polymerase chain reaction or by northern blot analysis normalised to glyceraldehyde 3-phosphate dehydrogenase or 18S rRNA, respectively. Three independent cultures were grown in each experiment, and each experiment was performed at least twice. Statistical comparisons were performed using the unpaired *t* test assuming equal variances. Data show that PGE_2 between 10nM and 1μM only modestly inhibits TGFβ1 induced CTGF expression. In contrast, PGE_2 completely blocked the stimulatory effect of TGFβ on CTGF levels in IMR-90 fibroblasts, as reported previously. These data were confirmed at the protein level by western blot analysis for CTGF. Forskolin treatment inhibited CTGF expression in both cell cultures, indicating that cAMP is inhibitory to CTGF expression. The protein kinase A inhibitor H-89 (10μM) did not block the effects of forskolin or PGE_2, indicating that protein kinase A does not mediate the downregulation of CTGF. Interestingly, inhibition of p38 mitogen activated protein kinase (MAPK) with SB-203580 resulted in sensitising gingival fibroblasts to the downregulation of CTGF by PGE_2, mimicking results in IMR-90 cells without a MAPK inhibitor. Stimulation of cAMP dependent Epac in IMR-90 cells presumably resulting in downstream inhibition of extracellular signal regulated kinase (ERK) 1/2 (p42/44 MAPK) increased CTGF expression, an effect not seen in gingival fibroblasts. These data indicate that p38 MAPK protects CTGF from PGE_2 dependent downregulation in gingival fibroblasts, whereas ERK 1/2 appears to confer PGE_2 downregulation of CTGF in IMR-90 cells. These data support the notion that tissue specific regulation of CTGF by PGE_2 in the presence of TGFβ1 contributes to the known tissue specificity of gingival overgrowth. Further analyses of these signal transduction pathways in primary human gingival fibroblasts, IMR-90 cells, human primary mesangial cells, and primary human dermal fibroblasts are in progress.

Additional studies are directed towards understanding the mechanisms by which CTGF promotes insoluble collagen deposition by human gingival fibroblasts. Data indicate that specific integrin neutralising antibodies, and certain anti-CTGF antibodies, inhibit the ability of CTGF to promote insoluble collagen deposition. These results provide the basis for a working hypothesis outlining the molecular interactions and signalling pathways initiated by CTGF that lead to increased collagen deposition in gingival cells.

O8 CCN1–INTEGRIN INTERACTIONS IN CELL SIGNALLING

C-C. Chen, V. Todorovic, N. Chen, F-E. Mo, L. F. Lau. *Department of Biochemistry and Molecular Genetics, University of Illinois College of Medicine, Chicago, IL 60607, USA*

CCN1 (CYR61), an archetypal member of the CCN family of secreted matricellular proteins, regulates diverse cellular functions including cell adhesion, migration, proliferation, survival, and differentiation. CCN1 also induces angiogenesis in vivo. Ccn1 null mice suffer embryonic death

as a result of defects in the cardiovascular system, thus establishing CCN1 as an important regulator of vascular development. Aberrant expression of CCN1 is associated with vascular diseases, such as atherosclerosis and restenosis, in addition to wound healing, arthritis, and breast cancer. Mechanistically, CCN1 acts through direct binding to integrin receptors and heparan sulfate proteoglycans. Although devoid of the RGD sequence motif, CCN1 is a direct ligand of at least five distinct integrins, including $\alpha_6\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_{IIb}\beta_3$, and $\alpha_M\beta_2$, which mediate CCN1 activities in a cell type and context specific manner. We have now identified multiple non-canonical binding sites in CCN1 for integrins $\alpha_6\beta_1$, $\alpha_v\beta_3$, and heparan sulfate proteoglycans. Moreover, we have constructed mutants in the context of full length CCN1 that are disrupted in each of the specific receptor binding sites and in combination. Current analysis of these integrin binding defective mutants is yielding new insights into the integrin specific CCN1 functions and signalling mechanisms. Novel activities of CCN1 and the signalling pathways that mediate them are being dissected using these integrin binding defective mutants.

O9 IDENTIFICATION OF RECEPTORS FOR CCN1 BY THE YEAST TWO HYBRID SYSTEM

P. M. Robertson, D. L. Simmons. *Department of Chemistry and Biochemistry, E280 Benson Science Building, Brigham Young University, Provo, UT 84602, USA*

The CCN family of genes has emerged as a group of regulators involved in important physiological processes such as neuronal differentiation, thrombosis, angiogenesis, wound healing, osteogenesis, and limb bud development. CCN genes are involved in cellular proliferation in general and specifically are either upregulated or downregulated in several cancers. CCN1 (Cyr61), a murine CCN member, was used as bait for screening a mouse embryo fibroblast library in the yeast two hybrid system to identify the cellular receptor(s) for CCN1, in addition to proteins involved in CCN1 associated physiological processes. Thirty different unique partial cDNA sequences have been identified, and one that was cloned five times was an exact match to mouse Notch 3. Other sequences obtained in our screen showed homology to other Notch genes, in addition to extracellular matrix proteins and other sequences with no DNA homology and others with no protein homology either. Each of the cDNA clones obtained is a reasonable candidate for interaction with CCN1 for two reasons. First, most of these proteins are characterised by either high cysteine residue content or the possession of at least one EGF repeat. Second, proteins (with homology to known proteins) that bound CCN1 have functional relevance, in that they are either known receptors, extracellular matrix proteins, or transcription factors (providing evidence for CCN1 in the nucleus).

O10 CCN2: INTERACTIONS WITH CELL SURFACE HEPARAN SULFATE PROTEOGLYCANS AND INTEGRINS ON FIBROGENIC CELL TYPES

R. Gao[1,2], D. Brigstock[1,2,3]. *[1]Center for Cell and Vascular Biology, Children's Research Institute, Columbus, OH, USA; [2]Department of Surgery and [3]Department of Molecular and Cellular Biochemistry, The Ohio State University, Columbus, OH, USA*

In the liver and the pancreas, the principal fibrogenic cell types are stellate cells. These cells are a relatively minor cell population in each organ and are ordinarily quiescent. During tissue injury, the cells undergo a process termed "activation", the result of which is that they become myofibroblastic, proliferative, motile, and produce a large amount of fibrillar collagens, which ultimately impede organ function. Stellate cells are so important in fibrotic pathways that emerging antifibrotic strategies will probably rely on the ability to prevent cell activation, to revert activated cells back to quiescent cells, and/or to initiate death of the activated cells.

Both pancreatic stellate cells (PSC) and hepatic stellate cells (HSC) produced CCN2 when activated in vitro. The production of CCN2 in these cells was also stimulated by transforming growth factor β (TGFβ), platelet derived growth factor, ethanol, and acetaldehyde. In contrast, other cell types such as hepatocytes, endothelial cells, and fibroblasts also produce CCN2 after injury to these organs, suggesting that the actions of CCN2 on stellate cells arise via complex, and probably overlapping, autocrine and paracrine pathways of stimulation. CCN2 stimulated proliferation, migration, adhesion, and matrix synthesis in both types of stellate cells. Stellate cell binding to CCN2 was functionally mediated by specific integrin subtypes in concert with heparin sulfate proteoglycan (HSPG) coreceptors. Whereas HSC used integrin $\alpha_v\beta_3$ for binding to CCN2, PSC used integrin $\alpha_5\beta_1$ instead. These respective integrin binding properties reside in module 4 and involve discrete non-overlapping domains. Stellate cell binding to CCN2 was heparin dependent, as shown by the fact that treatments such as preincubation of CCN2 with heparin, removal of cell surface HSPG by heparinase, or prevention of HSPG sulfation by sodium chlorate blocked the ability of CCN2 to support PSC or HSC adhesion.

Activation of integrin mediated pathways in stellate cells by CCN2 extended well beyond the phenomenon of cell adhesion. For example, migration of PSC in response to CCN2 was blocked by integrin antagonists or heparin. Moreover, gene array analysis of HSC showed that adhesive signalling increased transcription of profibrotic and antiapoptotic genes. Independent analysis confirmed that CCN2 supported nuclear factor κB dependent survival pathways in HSC and that CCN2 prevented apoptosis of activated HSC in an integrin dependent manner.

Collectively, these data suggest that the principal activities of CCN2 in stellate cells are to support an activated phenotype, specifically by driving proliferation, matrigenesis, and cell survival. Most, if not all, activities of CCN2 are probably mediated through specific integrin receptors and HSPG coreceptors. The central role of CCN2 in supporting stellate cell fibrogenesis and survival provides a sound rationale for targeting CCN2 or its receptors as a component of new antifibrotic strategies in the liver and pancreas.

O11 CCN2 MODES OF ACTION: MECHANISMS THAT MEDIATE MESANGIAL CELL DYSFUNCTION

N. Wahab, R. Mason. *Renal Section, Division of Medicine, Imperial College, School of Medicine, London, UK*

It is becoming clear that connective tissue growth factor (CTGF) is strongly implicated in the pathogenesis of diabetic nephropathy. However, the molecular mechanisms by which CTGF mediates mesangial cell dysfunction and thus the pathogenesis of this disease have not been elucidated. Our in vitro experiments provide evidence that in human mesangial cells (HMC), CTGF directly enhances the transforming growth factor β (TGFβ)/Smad signalling pathway in a mechanism that involves the transcriptional suppression of the Smad 7 gene, via induction of the immediate early gene TIEG, which encodes a repressor factor that binds to a specific element in the promoter of the Smad 7 gene and suppress its transcription.

Our experiments also showed that CTGF rapidly activates several intracellular signalling molecules, including extracellular signal regulated kinase (ERK) 1/2, ERK 5, JNK (Jun N-terminal kinase), protein kinase B (PKB), calmodulin dependent protein kinase type II, protein kinase Cα (PKCα), and PKCδ, suggesting that it functions via a signalling receptor. Crosslinking of recombinant CTGF to cell surface proteins with chemical cleavable crosslinker revealed that CTGF interacts with the dual receptor system, TrkA and $p75^{NTR}$. In support of this finding, the addition of recombinant CTGF to mesangial cells stimulated the phosphorylation of TrkA at tyrosine residues 490 and 674/675. K252a, a known selective inhibitor of Trks, blocked this phosphorylation, CTGF induced activation of signalling proteins and CTGF dependent induction of the transcription repressor TIEG.

We propose that CTGF functions via at least three different mechanisms. One is by regulating the TGFβ/Smad signalling pathway. Another is by its interaction with distinct tyrosine kinase receptors, triggering multiple intracellular signalling pathways. CTGF also seems to function through an intracellular transport pathway, in which it is internalised from the cell surface in endosomes then translocated into the nucleus, where it appears to interact with specific nuclear proteins.

O12 THE ROLE OF CCN4 AND CCN6 DURING CHONDROCYTE DIFFERENTIATION

K. Balakrishnan*, V. Church*, T. Newton, P. Francis-West. *Department of Craniofacial Development, King's College, London, UK*

We are interested in how chondrocyte differentiation is controlled with the aim of understanding how to repair both congenital and degenerate skeletal defects. Two candidate molecules are CCN4 and CCN6. Mutation of human CCN6 results in progressive pseudorheumatoid dysplasia, a syndrome characterised by the flaring of the epiphyses of the long bones, with ultimate joint loss during childhood, and flattening of the vertebrae.[1] This shows that CCN6 is essential for maintenance of the skeleton. CCN4 has been shown to function downstream of Wnt/β catenin signalling, a pathway that is also crucial for development and maintenance of the skeleton.[2] To investigate how these factors control chondrocyte differentiation, we have used two model systems: the developing chick limb bud and the in vitro ATDC5 cell line, which

undergoes progressive chondrocyte differentiation in culture. In situ hybridisation studies of developing chick limbs have shown that CCN4 is expressed in the developing joints and transiently in the chondrocytes of developing long bones, whereas localised expression of CCN6 is undetectable. However, CCN6 expression was detectable by reverse transcription polymerase chain reaction. In ATDC5 cells, CCN4 and CCN6 are expressed in differentiating but not undifferentiated cells. Gain of function studies have shown that CCN4 and CCN6 have distinct effects on chondrocyte differentiation, inhibiting and promoting matrix production, respectively. Inhibition is consistent with CCN6 being downstream of β catenin signalling. To understand these differences we have now begun analysis of the effect of the different domains. Preliminary results have shown that the first two domains can mimic the effect of the full length proteins. Finally, we have determined that CCN4 expression can be induced by LiCl, which activates the β catenin pathway, in undifferentiated ATDC5 cells.

*These authors contributed equally to this work.

1. **Hurvitz JR**, *et al. Nat Genet* 1999;**23**:94–8.
2. **Xu L**, *et al. Genes Dev* 2000;**14**:585–95.

O13 THE ROLES OF CCN2 IN SKELETAL REPAIR AND REGENERATION

M. Takigawa[1], T. Nishida[1], S. Kubota[1], S. Kojima[2], T. Kuboki[2], K. Nakao[1], T. Fujisawa[1], K. Kawata[1], T. Yanagita[1], H. Kadota[3], K. Asaumi[3], H. Kawaki[1], T. Kushibiki[4], Y. Tabata[4]. *[1]Department of Biochemistry and Molecular Dentistry, [2]Department of Oral and Maxillofacial Rehabilitation, [3]Department of Orthopaedic Surgery, Okayama University Graduate School of Medicine and Dentistry, Okayama 700–8525, Japan; [4]Institute for Frontier Medical Sciences, Kyoto University Graduate School of Medicine, Kyoto, 606–8507, Japan*

We previously reported that CCN2 promotes proliferation and differentiation of chondrocytes, osteoblasts, and endothelial cells. Because of the very high expression of the CCN2 gene in hypertrophic chondrocytes in the physiological state, we have suggested that a major physiological role for this factor is the promotion of endochondral ossification. However, recent observations of CCN2 gene expression during embryonic development and wound healing suggest the involvement of this factor in the growth and regeneration of various skeletal tissues. Here, we report on the role of CCN2 in skeletal repair and regeneration.

In a rat experimental osteoarthritis (OA) model and patients with OA, the clustered chondrocytes in articular cartilage (in which clustering indicates an attempt to repair the damaged cartilage) produced CCN2. Because CCN2 stimulates the proliferation and differentiation, but not hypertrophy, of articular chondrocytes, the role of CCN2 in cartilage repair was investigated. As a result, a single injection of CCN2 incorporated in gelatin hydrogel (CCN2-hydrogel) into the joint cavity of monoiodoacetate induced OA model rats repaired their articular cartilage to the extent that it became histologically and biochemically similar to normal articular cartilage. Next, to examine the effect of CCN2 on the repair of articular cartilage, we created defects (2 mm in diameter) on the surface of articular cartilage and then implanted CCN2-hydrogel therein with collagen sponge and found that new cartilage filled the defect four weeks postoperatively. Consistent with these in vivo effects, CCN2 enhanced type II collagen and aggrecan mRNA expression in mouse bone marrow derived stromal cells, and induced chondrogenesis in vitro. These findings suggest the utility of CCN2 in the regeneration of articular cartilage.

Macrophage colony stimulating factor induced CCN2 expression and proteoglycan synthesis in cultured chondrocytes, also suggesting crucial roles for CCN2 in cartilage repair during an inflammatory response.

In addition to gene expression and localisation of CCN2 during fracture healing, which we reported previously, we recently found gene expression and localisation of CCN2 during experimental distraction osteogenesis, further supporting the role of CCN2 in bone repair. Gene expression and localisation of CCN2 were also found during wound healing after tooth extraction, suggesting crucial roles in alveolar bone repair.

In many tissues/organs except cartilage, which is an avascular tissue, blood clotting is the first step of wound healing after injury. Because platelets contain abundant CCN2, CCN2 released from platelets may be important for the initiation of proliferation, migration, and adhesion of undifferentiated mesenchymal cells, perhaps including stem cells, to regenerate wounded tissues.

Molecular regulation of CCN2 for driving the proper regeneration of multiple types of tissues will be discussed.

O14 REGULATION OF SKELETAL DEVELOPMENT BY CCN1 AND CCN2

L. M. Dornbach[1], K. M. Lyons[1,2,3]. *Departments of [1]Biological Chemistry and [2]Molecular, Cellular, and Developmental Biology, University of California at Los Angeles, Los Angeles, CA, USA; [3]Department of Orthopedic Surgery, David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, CA, USA*

In the developing skeletal system, CCN1/Cyr61 and CCN2/CTGF are expressed in overlapping but distinct patterns in the perichondrium and growth plate of long bones. The expression patterns of CCN1 and CCN2 in cartilage, along with evidence that both of these genes can promote chondrogenesis in vitro, suggest that they are essential regulators of skeletal development. Knockouts for CCN1 and CCN2 have been described previously, and although $ccn2^{-/-}$ mice display generalised chondrodysplasia, mice lacking ccn1 die before chondrogenesis as a result of placental vascular insufficiency and weakened embryonic vasculature, precluding an analysis of ccn1 function in skeletogenesis. We are investigating the function of ccn1 in skeletal development and the possibility of functional redundancy between CCN1 and CCN2. By crossing the ccn1 mutation into different genetic backgrounds, we were able to recover some ccn1 mutants at midgestation stages and at birth (P0). At P0, $ccn1^{-/-}$ mice die perinatally of respiratory defects and display multiple abnormalities in the skeleton, including misalignment of the radius to the synovial joint, rib–sternum misalignment, sternal bifurcation, and defects in vertebral formation. We are currently investigating the molecular basis of these skeletal defects. We also have evidence of a genetic interaction between CCN1 and CCN2 in the regulation of skeletal development. Compound mutant ($ccn1^{+/-}$/$ccn2^{-/-}$) mice are phenotypically similar to $ccn2^{-/-}$ mice, but display additional defects in rib–sternum alignment and in the fusion of the posterior sternum. These sternal defects are never seen in $ccn1^{+/-}$ mice and are seen with only low penetrance in $ccn2^{-/-}$ mice. These results suggest that CCN1and CCN2 have both overlapping and unique functions in skeletal development.

O15 WISP-1 IS AN OSTEOBLASTIC REGULATOR EXPRESSED DURING SKELETAL DEVELOPMENT AND FRACTURE REPAIR

D. M. French[1], R. J. Kaul[2], A. L. D'Souza[2], C. W. Crowley[3], M. Bao[2], E. H. Filvaroff[2], G. D. Frantz[1], L. Desnoyers[1,2]. *Departments of [1]Pathology, [2]Molecular Oncology, and [3]Molecular Biology, Genentech Inc, South San Francisco, CA, USA*

CCN family members are involved in skeletogenesis and bone healing. To investigate the role of WISP-1 in osteogenic processes, we characterised its tissue and cellular expression and evaluated its activity in osteoblastic and chondrocytic cell culture models. During embryonic development, WISP-1 expression was restricted to osteoblasts and to osteoblastic progenitor cells of the perichondral mesenchyme. In vitro, WISP-1 expression in differentiating osteoblasts promoted bone morphogenetic protein 2 induced osteoblastic differentiation. In situ and cell binding analyses showed that WISP-1 interacts with perichondral mesenchyme and undifferentiated chondrocytes. We evaluated the effect of WISP-1 on chondrocytes by generating stably transfected mouse chondrocytic cell lines. In these cells, WISP-1 increased proliferation and saturation density but repressed chondrocytic differentiation. Because of the similarity between skeletogenesis and bone healing, we also analysed WISP-1 spatiotemporal expression in a fracture repair model. This model recapitulated the pattern of WISP-1 expression seen during skeletal development. These data show that WISP-1 is an osteogenic potentiating factor promoting mesenchymal cell proliferation and osteoblastic differentiation while repressing chondrocytic differentiation. Therefore, we propose that WISP-1 plays an important regulatory role during bone development and fracture repair.

O16 ROLE OF CCN3/NOV IN THE REGULATION OF BONE MARROW DERIVED STEM CELLS

K-i. Katsube. *Department of Molecular Pathology, Graduate School of Tokyo Medical and Dental University, Tokyo, Japan*

The application of bone marrow derived stem cells appears to be a promising technique for restitutive medicine, but the molecular mechanism of its regulation is still not clear. Notch signalling is important for the differentiation of stem cells, which is common in both vertebrates and invertebrates. We have used a mouse bone marrow derived stem cell line, Kusa, to analyse the role of Notch signalling. Principally, Kusa cells have a strong capacity for osteogenesis, but they can also differentiate

into neurones or other mesodermal tissues. Stable transfection of the constitutively active form of Notch downregulated osteogenesis but promoted neurogenesis in Kusa cells. However, using microarray technology, authentic signals downstream of Notch, such as the Hes gene family, or Notch ligands, such as Dll and Jag, were not activated in this transformant. We further investigated the microarray and found that some other basic helix–loop–helix genes such as MEF2C were upregulated. CCN3/Nov, a member of the CCN gene family can bind to the Notch EGF repeats to activate its signal. We found that CCN3/Nov expression was greatly upregulated by the constitutively active form of Notch and, in contrast, CCN3/Nov expression in Kusa stably transformed by CBF1/RBPJκ was an inhibitory nuclear factor of Notch signalling. Novel mechanisms of Nov/Notch signalling may underlie the differentiation of bone marrow derived stem cells. Some deletion constructs of the Nov protein are being applied to Kusa cells with the expectation that they will have a dominant negative effect on this hypothetical regulation.

O17 PARTICIPATION OF CTGF IN MUSCLE DIFFERENTIATION AND FIBROSIS AND ITS MODULATION BY PROTEOGLYCANS

C. Vial, V. Mezzano, P. Cañón, E. Brandan. *Centro de Regulación Celular y Patología, MIFAB, Facultad de Ciencias Biológicas, P. Universidad Católica de Chile, Santiago, Chile*

Duchenne muscular dystrophy (DMD) is characterised by degeneration of muscle fibres, which leads to progressive muscular atrophy. Regeneration of muscle fibres is dependent on the activation and proliferation of a population of progenitor cells termed satellite cells. In DMD, however, this process is insufficient to compensate for the continuous loss of myofibres and eventually fibrotic scarring ensues. At the same time, the deposition of fibrotic tissue precludes muscle healing and accelerates the course of the disease. Connective tissue growth factor (CTGF), which participates in normal skeletogenesis and angiogenesis, is also involved in numerous fibrotic disorders, but there is little information regarding its role in skeletal muscle disease. In addition, muscle differentiation is regulated by a variety of growth factors that inhibit or stimulate this process. Muscle differentiation is also finely regulated by proteoglycans that can present or sequester these growth factors. Our aim is to elucidate the role of CTGF in skeletal muscle biology at a cellular and molecular level in relation to its potential role in muscle differentiation and interaction with proteoglycans in this setting. Our second aim is to evaluate its participation in skeletal muscle fibrosis in DMD and its animal model the mdx mouse. Here, we report that transforming growth factor β (TGFβ), a profibrotic cytokine and potent inducer of CTGF expression in different biological scenarios, induces CTGF expression in the C2C12 mouse myoblast cell line, as does lysophosphatidic acid, another CTGF inducer molecule. We have generated a recombinant CTGF (rCTGF) protein capable of stimulating extracellular matrix (ECM) molecule synthesis in fibroblasts. Incubation of C2C12 with rCTGF stimulates their differentiation and the synthesis of ECM molecules. We have also found that rCTGF binds to proteoglycans such as decorin, which has the ability to bind TGFβ. Regarding animal physiology, CTGF expression is increased in fibrotic mdx compared with control mouse muscle tissue, as analysed by reverse transcription polymerase chain reaction. These results suggest that CTGF might be involved in the skeletal muscle fibrotic process and the differentiation of skeletal muscle, and may interact with proteoglycans.

Supported by FONDAP, MIFAB, and HHMI International Scholar.

O18 INDUCTION OF PULMONARY CTGF IN HEART FAILURE: EVIDENCE FOR INVOLVEMENT OF CTGF IN PULMONARY REMODELLING

M. S. Ahmed[1], E. Øie[1], T. von Lueder[1,2], L. E. Vinge[1], T. Attramadal[1], H. Attramadal[1,2]. *[1]Institute for Surgical Research, and [2]Department of Cardiology, Rikshospitalet University Hospital, University of Oslo, Oslo, Norway*

Pulmonary fibrosis and pulmonary arterial hypertension are complications of heart failure that add extra burden to the debilitated heart. Connective tissue growth factor (CTGF), a CCN family growth factor, is upregulated in fibrotic disorders of various aetiologies. To provide evidence of the involvement of CTGF in pulmonary remodelling in heart failure, investigations of pulmonary CTGF mRNA expression and protein content, in addition to analysis of the cellular distribution of pulmonary CTGF in a rat model of ischaemic heart failure, were performed. Pulmonary tissue samples of rats with ischaemic heart failure (three weeks after ligation of left coronary artery) and corresponding sham operated control rats were subjected to analysis of CTGF mRNA and protein concentrations by real time quantitative polymerase chain reaction and western blot analysis, respectively. CTGF mRNA values in pulmonary tissue of rats with heart failure were substantially raised compared with sham operated rats (fourfold increase compared with the sham group; $p < 0.05$), and corresponded with a similar increase in pulmonary CTGF content determined by immunoblot analysis. Furthermore, procollagen α1(I) mRNA values were also substantially raised in heart failure (1.9-fold increase compared with the sham operated rat; $p < 0.05$). Immunohistochemical analysis of the cellular distribution of CTGF in pulmonary tissue revealed increased anti-CTGF immunoreactivity in heart failure, with immunostaining predominantly of alveolar macrophages and endothelial cells. Alveolar macrophages aspirated by alveolar lavage of exsanguinated rats with heart failure and corresponding sham operated rats demonstrated robust increases of CTGF mRNA in heart failure. Histochemical staining with van Giessen revealed increased alveolar septal thickness with increased amounts of extracellular matrix. Pulmonary CTGF mRNA values also correlated with pulmonary dry weight ($R^2 = 0.71$; $p < 0.05$), a of measure pulmonary protein and extracellular matrix content. Intimal hyperplasia of pulmonary arteries was also detected, along with intimal fibroblast-like cells immuoreactive to anti-CTGF IgG.

In conclusion, this report demonstrates induction of pulmonary CTGF in heart failure, provided principally through activation of alveolar macrophages. Furthermore, induction of CTGF is associated with pulmonary and vascular remodelling, consistent with a role for CTGF in pulmonary fibrosis and pulmonary hypertension.

O19 CCN2 PROMOTES FIBROBLAST ADHESION TO FIBRONECTIN

Y. Chen[1], D. J. Abraham X. Shi-wen[11], C. M. Black[1], K. M. Lyons[2], A. Leask[1]. *[1]University College London (Royal Free Campus), London, UK; [2]University of California, Los Angeles, California, USA*

In vivo, CCN2 (connective tissue growth factor; CTGF) promotes angiogenesis, osteogenesis, tissue repair, and fibrosis, through largely unknown mechanisms. In vitro, CCN2 has been shown to promote cell adhesion in a variety of systems via integrins and heparin sulfate proteoglycans (HSPGs). However, the physiological relevance of CCN2 mediated cell adhesion is still unknown. We found that HSPGs and the MEK/extracellular signal regulated kinase (ERK) mitogen activate protein kinase cascade and are required for adult human dermal fibroblasts to adhere to CCN2. Endogenous CCN2 directly binds fibronectin and the fibronectin receptors, integrins $\alpha_4\beta_1$ and α_5 and syndecan 4. Using $Ccn2^{-/-}$ mouse embryonic fibroblasts, we found that loss of endogenous CCN2 results in impaired spreading on fibronectin, delayed α smooth muscle actin stress fibre formation, and reduced ERK and focal adhesion kinase phosphorylation. These results suggest that a physiological role of CCN2 is to potentiate the ability of fibroblasts to spread on fibronectin, which may be important in modulating fibroblast adhesion to the provisional matrix during tissue development and wound healing in vivo. These results are consistent with the notion that a principal function of CCN2 is to modulate receptor–ligand interactions.

O20 FUNCTION AND REGULATION OF CCN5 IN SMOOTH MUSCLE CELLS

J. Castellot. *Department of Anatomy and Cell Biology and Department of Pharmacology and Experimental Therapeutics, Tufts University School of Medicine, Boston MA, USA*

CCN5 is a secreted heparin and oestrogen regulated matricellular protein that has been shown to be an inhibitor of smooth muscle cell (SMC) proliferation and motility. In particular, we have shown that CCN5 suppresses both vascular SMC and human uterine SMC functions, including proliferation, motility, and matrix metalloproteinase 2 activity. Our laboratory is interested in exploring the pathophysiological roles of CCN5 and the mechanisms regulating its activity. To accomplish this goal, we are using several independent approaches that, taken together, should provide useful insights into CCN5 function. We had shown previously that oestrogen induced CCN5 in uterine tissues, but the effect of oestrogen on vascular SMC was not determined. We now have evidence that oestrogen does induce CCN5 expression in vascular SMC, with a concomitant inhibition of proliferation. As the first step in examining the downstream effectors of CCN5 action, we have begun gene microarray analysis. Initial results from human leiomyoma (fibroid) SMC suggest that a subset of extracellular matrix, membrane receptor, and cytoskeletal genes may be regulated by CCN5. We are also generating transgenic mice that will overexpress or underexpress CCN5

in an inducible, tissue specific manner. These mice, along with wild-type animals, will be subjected to both carotid ligation and wire injury procedures to examine the ability of CCN5 to suppress restenosis. We are also using humanised nude mice to assess the ability of CCN5 to prevent or reduce fibroidogenesis. These animal models will test the hypothesis that CCN5 is a useful therapeutic agent. Finally, we have examined the temporal and spatial expression patterns of CCN5 in embryonic development and in adult tissues. The unexpectedly large number of tissues and organs that express CCN5, combined with other data indicating that CCN5 is modified by tissue specific post-translational modification(s) in many tissues, suggest a wide range of potential functions that are cell and tissue specific.

O21 POTENTIAL ROLE OF CCN3 IN THE REGULATION OF GENE EXPRESSION

N. Planque[1], C. L. Li[1], S. Saule[2], A. Picard[1], L. Dassencourt[1], E. Kerbellec[1], B. Perbal[1]. [1]*Laboratoire d'Oncologie Virale et Moléculaire, UFR de Biochimie, Université Paris 7, 75005 Paris, France;* [2]*CNRS-UMR146, Institut Curie – Section Recherche, Centre Universitaire, Bâtiment 110, 91405 Orsay, France*

CCN3 was discovered as an integration site of the chicken retrovirus MAV in a kidney tumour. The integration of the proviral genome into the second intron of CCN3 resulted in the increased synthesis of an amino truncated form of the CCN3 protein. This protein morphologically transformed primary chicken embryonic fibroblasts in culture, whereas the secreted full length protein has antiproliferative activity in a variety of chicken and human cell lines. Because the lack of signal peptide was expected to alter the fate of the truncated protein, we hypothesised that modifications of CCN3 subcellular addressing could uncover potential oncogenic activities. Nuclear staining was detected by immunofluorescence with antibodies targeted against the CT domain of CCN3 in human HeLa, osteosarcoma 143, choriocarcinoma Jeg3, and in rat glioma C6 cancer cell lines. Western blot analysis identified nuclear CCN3 proteins with a molecular mass of 28–30 kDa in several cancer cells.

To study the mechanisms governing subcellular addressing of the different CCN3 proteins, we constructed a series of plasmids encoding the full length and truncated CCN3 proteins fused to autofluorescent tags. The results obtained established that the CT domain of CCN3 directs the chimaeric proteins into the nucleus.

In a yeast two hybrid assay, CCN3 was previously shown to interact with the rbp7 subunit of RNA polymerase II. Furthermore, immunocytochemistry revealed that, in 143 cells, CCN3 colocalised with the transcription machinery but not with the replication machinery. To study the putative role of CCN3 in gene transcription, we have constructed plasmids expressing chimaeric proteins in which CCN3 modules or combinations of modules are fused in frame with the DNA binding domain of Gal4. Our results identified the nuclear CCN3 proteins as regulators of transcription.

Possible mechanisms governing the nuclear translocation and the potential functions of truncated isoforms in gene expression will be discussed.

O22 CCN3 INTERACTIONS WITH CONNEXIN43: GAP JUNCTIONS AND GROWTH CONTROL

C. C. Naus[1], C. T. Fu[1], J. F. Bechberger[1], M. A. Ozog[1], B. Perbal[2]. [1]*Department of Anatomy and Cell Biology, University of British Columbia, Vancouver V6T 1Z3, British Columbia, Canada;* [2]*Laboratoire d'Oncologie Virale et Moléculaire, UFR de Biochimie, Université Paris 7-D. Diderot, 75005 Paris, France*

Many tumour cells exhibit aberrant gap junctional intercellular communication that can be restored by transfection with connexin genes. We previously discovered that overexpression of connexin43 (Cx43) in C6 glioma cells not only reduces proliferation, but also leads to the production of soluble growth inhibitory factors. We found that several members of the CCN (Cyr61/connective tissue growth factor/nephroblastoma overexpressed) family are upregulated after Cx43 expression, including CCN3 (NOV). We now report evidence for an association between CCN3 and Cx43. Western blot analysis demonstrated that the 48 kDa full length CCN3 protein was present in the lysate and conditioned medium of growth suppressed C6-Cx43 cells and in primary astrocytes, but not in C6 parental and human glioma cells. Immunocytochemical examination of CCN3 revealed diffuse localisation in parental C6 cells, whereas transfection of C6 cells with Cx43 (C6-Cx43) or with a modified Cx43 tagged to green fluorescent protein at its C-terminus (Cx43-GFP) resulted in punctate staining, suggesting that CCN3 colocalises with Cx43 in plaques at the plasma membrane. In cells expressing a C-terminal truncation of Cx43 (Cx43Δ244–382), this colocalisation was lost. Glutathione-S-transferase pulldown assays and co-immunoprecipitation showed that CCN3 could physically interact with Cx43. In contrast, CCN3 did not associate with Cx43Δ244–382. Similar experiments revealed that CCN3 did not colocalise or associate with other connexins, including Cx40 and Cx32. Taken together, these data support an interaction of CCN3 with the C-terminus of Cx43, which could play an important role in mediating growth control induced by specific gap junction proteins.

O23 CONNECTIVE TISSUE GROWTH FACTOR MEDIATES HIGH GLUCOSE EFFECTS ON MATRIX DEGRADATION THROUGH TISSUE INHIBITOR OF MATRIX METALLOPROTEINASE TYPE 1: IMPLICATIONS FOR DIABETIC NEPHROPATHY

S. V. McLennan[1,2], X. Wang[1], V. Moreno[2], D. K. Yue[1,2], S. M. Twigg[1,2]. [1]*Discipline of Medicine, University of Sydney, Sydney, Australia;* [2]*Department of Endocrinology, Royal Prince Alfred Hospital, Sydney, NSW, 2006 Australia*

High glucose concentrations inhibit matrix degradation and affect the activities of the enzymes responsible, the matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs). The expression of connective tissue growth factor (CTGF; also known as ccn2), which is a downstream mediator of transforming growth factor β (TGFβ), is increased in diabetic nephropathy. However, it is still unclear whether CTGF regulates matrix degradation and the mechanism of its effect in diabetes is still unknown. Human mesangial cells were cultured in medium containing 5mM or 25mM glucose, and in some experiments, with recombinant human CTGF (rhCTGF; 0–1000 ng/ml) and/or appropriate neutralising antibodies. Matrix degradation was inhibited by rhCTGF in a dose dependent manner, and the decrease in matrix degradation caused by high glucose and by TGFβ1 was significantly attenuated by the addition of CTGF neutralising antibody (by 40.2% and 69.1%, respectively). Similar to 25mM glucose, the addition of rhCTGF increased MMP-2, TIMP-1, and TIMP-3 mRNA 2.5, 2.1, and 1.6-fold, respectively ($p < 0.05$), but had no effect on MT1-MMP or TIMP-2. The addition of anti-TIMP-1 antibody to conditioned medium abolished the decrease in degradation caused by rhCTGF and partially prevented (by 79%) the glucose induced inhibition of matrix degradation. In vivo studies of glomeruli from diabetic and control rats showed that intensive insulin treatment prevented the increase in expression of CTGF and TIMP-1 and attenuated the decreased matrix degradation seen in diabetes. In summary, CTGF inhibits matrix degradation by increasing TIMP-1 expression, and by this action it contributes to the inhibition of matrix breakdown by high glucose, implying that CTGF has a role in the reduced matrix degradation seen in diabetic nephropathy.

Supported by National Health and Medical Research Council of Australia and Diabetes Australia Research Trust.

O24 USE OF A FOCUSED CDNA MICROARRAY TO INVESTIGATE THE ROLE OF CCN2 IN EXTRACELLULAR MATRIX PRODUCTION AND ACTIN CYTOSKELETON REARRANGEMENT IN A MESANGIAL CELL MODEL OF DIABETIC NEPHROPATHY

R. J. O'Leary[1], J. K. Crean[2], F. Furlong[1], F. Martin[1]. [1]*Departments of Pharmacology and* [2]*Medicine and Therapeutics, Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland*

Introduction: Hyperglycaemia induced increases in glomerular mesangial extracellular matrix production and actin cytoskeleton rearrangement are key pathological hallmarks of diabetic nephropathy. Previously, we have described the use of a suppressive subtractive hybridisation (SSH) screen to identify mRNA transcripts, which are differentially expressed in human glomerular mesangial cells (HMCs) propagated in vitro under conditions of either normal (5mM) or high (30mM) physiological glucose. In this study, we have used focused cDNA microarray technology to characterise the expression profile of genes derived from this SSH in HMCs treated with transforming growth factor β1 (TGFβ1) and connective tissue growth factor (CTGF/CCN2).

Materials/Methods: From the SSH screen, 171 distinct clones were amplified via the polymerase chain reaction (PCR) and arrayed on to glass slides. Primary HMCs were cultured under standard conditions and incubated for 24 hours with either TGFβ1 or CCN2. cDNA was synthesised from the resultant total RNA and labelled with one of the fluorescent dyes, Cy3 or Cy5. Experimental hybridisations were carried

out by competing control RNA against RNA from a stimulated state in each of the cases. Furthermore, HMCs were treated with CCN2 and cytoskeletal organisation was investigated by immunocytochemistry and western blot analysis.

Results: Analysis of our focused microarray in response to stimulation with TGFβ1 revealed an increase in the fluorescent intensities for 11 genes including lysyl oxidase (LOX), matrix metalloproteinase 1 (MMP1), actin regulatory protein 3 (ARP3), and the GTPase activator ASAP1. TGFβ1 also induced the expression of two key marker genes for mesangial cell dysfunction—fibronectin and CCN2. The increase in expression of these two marker genes was validated using real time PCR. Furthermore, an increase in the protein concentration of both fibronectin and CCN2 in response to TGFβ1 stimulation was demonstrated via western blotting. Analysis of the focused microarrays investigating HMCs stimulated with CCN2 revealed induction of 10 distinct transcripts. CCN2 induced the expression of the actin/myosin binding protein caldesmon, along with ARP3 and fibronectin. In addition, CCN2 caused the downregulation of tubulin α3 and the F-actin capping protein. HMCs treated with CCN2 demonstrated disassembled F-actin filaments with a concurrent increase in RhoA activity. When HMCs were stimulated with CCN2 and stained for tubulin, widespread microtubular rearrangement was seen at four hours.

Conclusion: CCN2 mediated actin rearrangement may contribute to the pathophysiology of the glomerular mesangium in diabetic nephropathy.

O25 CONNECTIVE TISSUE GROWTH FACTOR EXPRESSION AND DISTRIBUTION IN DIFFERENT STAGES OF ATHEROSCLEROTIC PLAQUE DEVELOPMENT

I. Cicha[1], A. Yilmaz[1], W. G. Daniel[1], D. R. Brigstock[3], M. Goppelt-Struebe[2], C. D. Garlichs[1]. *[1]Medical Clinic II, [2]Medical Clinic IV, University of Erlangen-Nuremberg, Erlangen, Germany; [3]Center for Cell and Vascular Biology, Children's Research Institute, Columbus, Ohio, USA*

Connective tissue growth factor (CTGF) mRNA is expressed at very high levels in atherosclerotic blood vessels, but the role of CTGF in atherosclerosis remains controversial. Despite its matrix inducing effect, CTGF is thought to contribute to plaque progression by enhancing platelet and monocyte adhesion to dysfunctional endothelium, and by promoting smooth muscle cell apoptosis. To elucidate the possible role of CTGF in atherosclerosis, we investigated CTGF protein expression and distribution in different stages of plaque development. Serial cross sections of 50 human carotid plaques were immunohistochemically analysed for the presence of CTGF protein, von Willebrand factor, macrophages (CD68), and T cells (CD3). According to their morphologies, plaques were defined as initial lesions, fibrous advanced lesions, or lipid rich advanced plaques. Initial lesions contained very few CTGF expressing cells. This finding was subsequently confirmed using in situ hybridisation. In advanced plaques, CTGF levels were significantly higher in lipid rich compared with fibrous plaques ($p < 0.004$). CTGF accumulated particularly in the rupture prone plaque shoulder, and in the areas of neovascularisation and/or infiltration with inflammatory cells. Independent of plaque morphology, a significant reduction in CTGF was found in patients treated with statins ($p = 0.01$). There were no significant correlations between mean CTGF positive cell numbers and smoking, hyperlipidaemia, or diabetes mellitus. Macrophage-like cells stained positive for CTGF protein in plaques. Subsequent in vitro studies showed that although activated monocyte derived macrophages do not produce CTGF, they can take it up from culture medium. Furthermore, CTGF acts as a chemoattractant for human mononuclear cells. In conclusion, CTGF protein is significantly increased in lipid rich compared with fibrous plaques, and may enhance monocyte migration into atherosclerotic lesions, thus contributing to atherogenesis.

O26 TARGETING CCN2 IN THE DIAGNOSIS AND TREATMENT OF PROGRESSIVE RENAL FIBROSIS

B. L. Riser. *Baxter Healthcare, Renal Division, and Rosalind Franklin University of Medicine and Science Department of Physiology and Biophysics, McGaw Park, Illinois, USA*

Background: It is currently not possible to predict those patients with diabetes who will develop diabetic nephropathy (DN) and progress to kidney failure. Patients are presently monitored for microalbuminuria, as a sign of overt clinical renal disease. However, microalbuminuria is not a predictor, but in fact an indicator of established glomerular damage. Patients with confirmed microalbuminuria are then typically started on treatments that retard, but do not block or reverse progression. Glomerular and interstitial expression of CCN2 is greatly upregulated early in experimental and in human diabetes. Further mesangial cell exposure to CCN2 increases its production of extracellular matrix molecules responsible for glomerulosclerosis. Although a blockade of CCN2 in diabetes to demonstrate a casual role for CCN2 has not yet been accomplished, it has been in other short term models of kidney fibrosis. It has been postulated that the early presence of CCN2 in urine is predictive of the later development of renal pathology associated with chronic kidney disease, and that the ability to predict DN should allow improved efficacy of treatment. This study tests this first hypothesis in both human and experimental diabetic renal disease.

Methods: Urine samples from (1) healthy rats, (2) rats made diabetic by streptozotocin (STZ), (3) healthy human volunteers, (4) patients with diabetes and renal disease, and (5) patients with diabetes without renal disease were examined by western blotting and/or enzyme linked immunosorbent assay for qualitative and quantitative analysis of CCN2.

Results: Low levels of urinary CCN2 were present in healthy, control rats, but were increased approximately sevenfold overall in STZ diabetic animals. CCN2 values were the highest at week 3 of diabetes then decreased with time, but remained significantly raised compared with controls, even after 32 weeks. Consistently low concentrations of urinary CCN2 were also detected in healthy volunteers (mean value, 7.1 CCN2/mg creatinine). However, concentrations were increased approximately sixfold in most of the patients with diabetes and nephropathy. A small number of the patients with diabetes not yet exhibiting evidence of renal involvement had urinary CCN2 concentrations that were ninefold greater than controls. The remaining normoalbuminuric patients with diabetes had CCN2 values that were indistinguishable from those of healthy volunteers. Analysis by western blotting confirmed the identity of the urinary CCN2. A molecular species equivalent to full length CCN2 (37/39 kDa doublet) was present in healthy controls. In contrast, the nephropathic group showed multiple CCN2 bands.

Conclusions: These findings support the hypothesis that CCN2 is upregulated early in DN. Urinary CCN2 could be useful to help stage nephropathy and predict those patients who are destined to develop DN. Because renal fibrosis/sclerosis in chronic renal disease appears to involve well defined mechanisms, almost independent of the initial insult, determination of quantitative and qualitative expression of CCN2 may also prove useful for early diagnosis, and determination of therapeutic efficacy in renal fibrosis where diabetes is not the cause.

O27 CTGF PLASMA CONCENTRATIONS CORRELATE WITH PLASMA CREATININE CONCENTRATIONS IN PATIENTS WITH TYPE 1 DIABETES MELLITUS

P. Roestenberg[1], L. Tarnow[2], L. Wieten[1], N. Oliver[3], W. Usinger[3], H. H. Parving[2], R. Goldschmeding[1], F. A. van Nieuwenhoven[1]. *[1]Department of Pathology, UMCU, Utrecht, The Netherlands; [2]Steno Diabetes Centre, Gentofte, Denmark; [3]Fibrogen Inc, South San Francisco, CA, USA*

Connective tissue growth factor (CTGF) is a 36–38 kDa protein strongly upregulated in fibrotic disorders including diabetic nephropathy (DN). Plasma CTGF concentrations and urinary CTGF excretion are increased in both human and experimental DN. Furthermore, plasma CTGF concentrations in patients with type 1 diabetes were correlated with albuminuria and creatinine clearance. The aim of the present study was to investigate the possible association of plasma CTGF concentrations with markers of DN in a larger cross sectional study of patients with type 1 DM.

Three hundred and eighty seven patients with type 1 DM were included in the study. One hundred and ninety nine patients had DN, which was clinically defined as urinary albumin excretion rate (UAER) > 300 mg/24 hours in at least two of three consecutive 24 hour urine collections, presence of retinopathy, and no evidence of other kidney disease. One hundred and eighty eight patients with DM and normoalbuminuria (NA), who were matched for sex, age, and duration of DM, were included as controls. Plasma CTGF concentrations were determined by sandwich enzyme linked immunosorbent assay. Stepwise regression analysis was used to analyse the possible correlation between plasma CTGF concentrations and relevant patient characteristics.

Plasma CTGF values were significantly higher in patients with DN (mean, 20 ng/ml; SD, 17) than in those with NA (mean, 10 ng/ml; SD, 6). When all 387 patients were included, stepwise regression analysis revealed a correlation between log plasma CTGF and log plasma creatinine values ($R = 0.60$; $p < 0.001$). In addition, systolic blood pressure, log UAER, age, and body mass index also contributed as predictors of CTGF plasma concentration (cumulative $R = 0.64$). When subgroups of patients were analysed separately, the correlation between log plasma CTGF and log plasma creatinine values was present in the DN group ($R = 0.65$; $p < 0.001$) but absent in patients with NA ($R = 0.018$; $p = 0.81$).

Thus, CTGF plasma concentrations are increased in patients with type 1 DM with DN, and plasma CTGF correlates with plasma creatinine values and albuminuria. The relative contributions of decreased filtration and increased production of CTGF in patients with DN remain to be established.

O28 CONNECTIVE TISSUE GROWTH FACTOR IS ASSOCIATED WITH FIBROSIS IN VITREORETINAL DISORDERS IN THE HUMAN EYE

E. J. Kuiper[1], M. D. de Smet[1], J. C. van Meurs[2], H. S. Tan[1], M. W. Tanck[3], N. Oliver[4], F. A. van Nieuwenhoven[5], R. Goldschmeding[5], R. O. Schlingemann[1]. *[1]Ocular Angiogenesis Group, Department of Ophthalmology, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands; [2]Department of Ophthalmology, Rotterdam Eye Hospital, Rotterdam, The Netherlands; [3]Department of Clinical Epidemiological Statistics, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands; [4]FibroGen, 225 Gateway Boulevard, South San Francisco, California, USA; [5]Department of Pathology, University Medical Centre Utrecht, Utrecht, The Netherlands*

Background: Fibrosis and scarring lead to irreparable loss of vision in several major diseases of the retina. Fibrosis is also the unpreventable outcome of ocular neovascularisation.

Aims: Connective tissue growth factor (CTGF), an inducer of fibrosis, and the angiogenesis factor vascular endothelial growth factor (VEGF) were investigated in relation to fibrosis and neovascularisation in human vitreoretinal disorders in an attempt to identify factors involved in ocular fibrosis that may serve as new therapeutic targets.

Methods: Vitreous samples were obtained from 53 patients with vitreoretinal disorders associated with varying degrees of fibrosis: proliferative diabetic retinopathy (PDR), proliferative vitreoretinopathy, and epiretinal membrane and macular hole. Clinical data, including the degree of intraocular fibrosis and neovascularisation, were collected using standardised forms. The concentrations of CTGF and VEGF were measured by enzyme linked immunosorbent assay.

Results: Significant univariate associations were found between CTGF and the degree of fibrosis, degree of neovascularisation, diabetes, and VEGF. Multivariate analysis showed that degree of fibrosis ($p = 0.01$) and VEGF ($p = 0.02$) are the best predictors of a rise in CTGF concentrations in the human vitreous humour (together $R^2 = 43.6\%$). Moreover, CTGF concentration predicts the degree of fibrosis ($p = 0.001$). In the 10 patients with PDR and neovascularisation, the ratio of CTGF to VEGF strongly correlated with the degree of fibrosis ($\rho = 0.661$; $p = 0.04$).

Conclusions: CTGF may be an important causal factor in ocular fibrosis, and an increased CTGF : VEGF ratio may be crucial in the angiofibrotic switch occurring in the course of ocular angiogenesis. CTGF may therefore represent a suitable therapeutic target for prevention of sight threatening vitreoretinal scarring.

O29 CCN3 EXPRESSION IS REDUCED AS A RESULT OF BCR–ABL KINASE IN CHRONIC MYELOID LEUKAEMIA

L. M. R. Gilmour[1], S. Price[1], A. Kwasniewska[1], A. Pierce[2], A. D. Whetton[2], B. Perbal[3], A. E. Irvine[1]. *[1]Department of Haematology, Queen's University, Belfast, Northern Ireland, UK; [2]LRF Cellular Development Unit, UMIST, Manchester, UK; [3]Laboratoire d'Oncologie Virale et Moléculaire, UFR de Biochimie, Université Paris 7 D. Diderot, France*

Chronic myeloid leukaemia (CML) is characterised by expression of the constitutively active Bcr–Abl tyrosine kinase. Downregulation of CCN3, both mRNA and protein, has been demonstrated as a result of Bcr–Abl kinase expression in a murine CML cell line model. This present study demonstrates reduced expression of CCN3 in human CML cell lines and primary human CML cells and shows modulation of CCN3 expression in response to Bcr–Abl kinase.

Real time polymerase chain reaction was used to monitor mRNA expression of Bcr–Abl and CCN3. The human CML cell lines K562, KU812, and LAMA showed strong expression of Bcr–Abl, whereas CCN3 was barely detectable. Treatment of human CML cells from the K562 cell line with the Bcr–Abl kinase inhibitor, Imatinib (1μM; two to four days), resulted in a reduction of Bcr–Abl expression and a concomitant increase in CCN3 expression (mean Ct change: Bcr–Abl, 2.0 (SD, 0.6); CCN3, 2.3 (SD, 0.3)). Similarly, treatment of K562 cells with si-Bcr–Abl (small interfering-RNA directed at Bcr–Abl) confirmed that decreasing Bcr–Abl expression resulted in increased expression of CCN3 (minimal Ct change: Bcr–Abl, 2.0; CCN3, 3.0). Bone marrow was obtained from three normal donors and from three patients with CML at both diagnosis and on entering cytogenetic remission. Primary human CML cells at diagnosis showed a four times lower expression of CCN3 mRNA compared with normal bone marrow samples (NBM) ($p = 0.015$). Bcr–Abl expression fell significantly when patients entered haematological remission and there was a reciprocal increase in CCN3 expression ($p = 0.026$; mean CCN3 Ct change, 1.0; SD, 0.1). Confocal microscopy and western blotting showed analogous changes in CCN3 protein expression, with a reversion to levels comparable with NBM upon entering remission.

Dysregulation of CCN3 expression has not previously been recognised in CML. CCN3 and Bcr–Abl expression appear to be inversely related. Loss of CCN3 expression is consistent with the CML phenotype.

O30 A NOVEL CYR61 TRIGGERED ''CYR61–$\alpha_v\beta_3$ INTEGRIN LOOP'' REGULATES BREAST CANCER CELL SURVIVAL AND CHEMOSENSITIVITY THROUGH ACTIVATION OF ERK1/ERK2 MAPK SIGNALLING PATHWAY

R. Lupu, L. Vellon, J. A. Menendez. *Department of Medicine, Breast Cancer Translational Research Laboratory, Evanston Northwestern Healthcare Research Institute and Northwestern University Feinberg School of Medicine, Chicago, USA*

The angiogenic inducer CYR61 is differentially overexpressed in breast cancer cells exhibiting high levels of heregulin (HRG), a growth factor closely associated with a metastatic breast cancer phenotype. This study examined whether CYR61, independently of HRG, actively regulates breast cancer cell survival and chemosensitivity, and the pathways involved. Forced expression of CYR61 in HRG negative MCF-7 cells notably upregulated the expression of its own integrin receptor $\alpha_v\beta_3$ ($>$ 200 times). Small peptidomimetic $\alpha_v\beta_3$ integrin antagonists dramatically decreased the cell viability of CYR61 overexpressing MCF-7 cells, whereas control MCF-7/V remained insensitive. Mechanistically, functional blockade of $\alpha_v\beta_3$ specifically abolished CYR6 induced hyperactivation of extracellular signal regulated kinase 1 (ERK1)/ERK2 mitogen activated protein kinase (MAPK), whereas the activation status of AKT did not decrease. Moreover, CYR61 overexpression rendered MCF-7 cells significantly resistant ($>$ 10-fold) to Taxol induced cytotoxicity. Remarkably, $\alpha_v\beta_3$ inhibition converted the CYR61 induced Taxol resistant phenotype into a hypersensitive one. Thus, the augmentation of Taxol induced apoptotic cell death in the presence of $\alpha_v\beta_3$ antagonists demonstrated strong synergism, as verified by the terminal transferase mediated dUTP nick end labelling assay and by flow cytometric analysis for DNA content. Indeed, functional blockade of $\alpha_v\beta_3$, similarly to the pharmacological MAPK inhibitor U0126, synergistically increased both the proportion of CYR61 overexpressing breast cancer cells in the G_2–M phase of the cell cycle and the appearance of sub-G_1 hypodiploid (apoptotic) cells caused by Taxol. Strikingly, CYR61 overexpression impaired the accumulation of wild-type p53 after Taxol exposure, whereas $\alpha_v\beta_3$ and ERK1/ERK2 MAPK blockade completely restored Taxol induced upregulation of p53. Moreover, antisense downregulation of CYR61 expression abolished the anchorage independent growth of breast cancer cells engineered to overexpress HRG, and significantly increased their sensitivity to Taxol. These data provide evidence that CYR61 is sufficient to promote breast cancer cell proliferation, cell survival, and Taxol resistance through $\alpha_v\beta_3$ activated ERK1/ERK2 MAPK signalling. The identification of a ''CYR61–$\alpha_v\beta_3$ autocrine loop'' in the epithelial compartment of breast carcinoma strongly suggests that targeting $\alpha_v\beta_3$ may simultaneously prevent breast cancer angiogenesis, growth, and chemoresistance.

O31 CCN1 (CYR61): TWO FACES IN CANCER

H. P. Koeffler. *Department of Medicine, Hematology/Oncology Division, Cedars-Sinai Medical Center, University of California, Los Angeles, Los Angeles, CA USA*

Cyr61 (CCN1) can stimulate the growth of selected tumours and inhibit the growth of others. Breast cancers, glioblastoma multiforme (brain tumours), and ovarian cancers frequently overexpress Cyr61. In contrast, endometrial and lung cancers often have low expression of Cyr61. Forced overexpression of Cyr61 in breast, brain, and ovarian cancers stimulates their growth in vitro and in immunodeficient mice. This growth is associated with prominent angiogenesis. Use of small interfering RNA (siRNA) Cyr61 inhibits the growth of these cancer cells. Examination of the signalling pathways suggest that overexpression of Cyr61 in these cells can activate the integrin link kinase to stimulate the β-catenin–TCF (T cell factor)/lymphocyte enhancing factor (LEF) complex to enhance expression of proliferation related genes. In addition, at least in gliomas cells, the phosphoinositol 3 kinase/AKT pathway is activated. Wild-type p53 is overexpressed in breast cancer cells that have forced,

high levels of Cyr61 (MCF-7), but paradoxically, this proapoptotic gene is non-functional in these cells. Furthermore, breast cancer cells (MCF-7) that have forced expression of four deletional mutants of Cyr61 (missing one of its four structural motifs), have only subtle growth defects in vitro; however, each of these motifs appears to be important for robust tumour formation of these breast cancer cells in nude mice. In contrast, overexpression of Cyr61 in endometrial or lung cancer cells decreases their growth, with an increase in apoptosis associated with raised expression of Bax, Bad, and TRAIL. This inhibition of growth appears to be p53 independent in endometrial cancer cells; but in lung cancer cells, p53 helps mediate the growth suppression. Use of siRNA Cyr61 in endometrial cancer cells stimulates their proliferation and decreases apoptosis. In another series of experiments, many of the findings for Cyr61 in the above cancers also applied to CTGF. In summary, overexpression of Cyr61 occurs in breast, ovary, and brain cancers and is associated with growth stimulation in these cells. In contrast, many endometrial and lung cancers have low expression of Cyr61 and forced expression of Cyr61 in these tumour cells is associated with growth inhibition as a result of increased apoptosis.

Poster presentations

P1 TEMPORAL AND SPATIAL LOCALISATION OF CCN5 PROTEIN IN MURINE EMBRYOS

J. Jones, M. Blea, M. Sullivan, J. Castellot, M. Gray. *Department of Anatomy and Cell Biology, Tufts University School of Medicine, Boston MA, USA*

CCN5 is highly conserved among vertebrates and is the only CCN family member lacking the C-terminal domain. CCN5 distribution in embryos has not been mapped either spatially or temporally in a systematic manner. Two separate observations present a challenge in attempting to understand the physiological roles and mechanisms of action of CCN5. In the adult, CCN5 is a strong inhibitor of smooth muscle cell (SMC) proliferation and displays the characteristics of a growth arrest specific gene. However, in the uterus, CCN5 mRNA and protein values increase dramatically upon exposure to oestrogen. These observations lead to paradoxical predictions of CCN5 expression patterns in the developing embryo: the antiproliferative action of CCN5 predicts that it should not be present in an embryonic tissue until the proliferation phase of the morphogenesis of that tissue is complete; however, embryos are bathed in high concentrations of oestrogen, which predicts strong CCN5 expression throughout the embryo. To examine this conundrum, 7 μm thick frozen sections from fixed murine embryos from 8 to 18 days post coitum were analysed by immunohistochemistry using an affinity purified, antipeptide polyclonal antibody directed against a CCN5 specific peptide. CCN5 was present in nearly all embryo tissues at the stages analysed. Expression was particularly strong in vascular tissues, cardiac muscle, skeletal muscle primordia, brain and spinal cord, digestive tract, and respiratory tract. Expression was absent in cartilage and high in osteoblasts, in direct contrast to the expression pattern seen for CCN2. These data suggest that the ubiquitous embryonic distribution of CCN5 might be caused by widespread gene expression stimulated by high concentrations of oestrogen exposure in utero. The presence of high levels of CCN5 even in proliferating tissues suggests that it functions in a highly cell and tissue specific context.

P2 LOCALISATION OF CCN5 PROTEIN IN ADULT MURINE TISSUES

M. Blea[1], M. Sullivan[1], J. Jones[1], L. Oleson[2], M. Gray[1], J. Castellot[1,2]. [1]*Department of Anatomy and Cell Biology and* [2]*Department of Pharmacology and Experimental Therapeutics, Tufts University School of Medicine, Boston MA, USA*

CCN5 is a secreted heparin and oestrogen regulated matricellular protein that has been shown to be an inhibitor of smooth muscle cell proliferation and motility. In an accompanying poster, we show that CCN5 is generally expressed throughout the embryonic development of the mouse in many organs and tissues. We have hypothesised that this results from the hyper-oestrogenic environment of the embryo. However, after embryonic development is complete, we predict that CCN5 distribution will be largely restricted to smooth muscle tissues—for example, those in the large blood vessels, uterus, airways, and digestive tract. To test this hypothesis, adult organ and tissue samples from male and female mice and rats were isolated, fixed rapidly postmortem, and frozen at −70˚C. Frozen sections (7 μm thick) were analysed by immunohistochemistry using an affinity purified, antipeptide polyclonal CCN5 antibody. Intense CCN5 expression was detected in the heart muscle, aorta, uterus, bronchi, and digestive tract organs, as expected. However, CCN5 was also detected in many other adult tissues analysed, including the pancreas, spleen, skeletal muscle, ovary, testis, thymus, brain, and kidney. Although CCN5 expression was often found in the smooth muscle-like tissues within an organ (for example, in the mesangial cells of the kidney glomerulus), strong expression of CCN5 was also seen in epithelial tissues. In particular, expression in the epithelial lining of ducts in the kidney and uterine endometrium was especially striking. The distribution of CCN5 in adult organs and tissues suggests that CCN5 might have additional biological functions beyond those previously identified in vascular and uterine smooth muscle cells.

P3 ANALYSIS OF TISSUE SPECIFIC CCN5 VARIANTS

M. Gray, J. Castellot. *Department of Anatomy and Cell Biology, Tufts University School of Medicine, Boston MA, USA*

CCN5 regulates the proliferation and motility of vascular and uterine smooth muscle cells, and is widely expressed in embryonic and adult tissues as assessed by immunohistochemistry (IHC). Using western blot analysis, a 28 kDa CCN5 band was expected in lysates made from all adult tissues that show the presence of CCN5 by IHC using an affinity purified, antipeptide, CCN5 specific antibody. The intensity of the CCN5 band was expected to be proportional to the IHC staining intensity. To test this hypothesis, adult male and female organs from mouse and rat were carefully removed and separated from surrounding tissues immediately postmortem and frozen at −70˚C. Tissue samples were minced and cells were disrupted using a standard lysis protocol. Tissue lysates were stored at −20˚C until analysis on western blots using PVDF membranes. Although a 28 kDa band was detected in most of the tissues analysed, a 47 kDa band was present in all tissues examined, including aorta, uterus, heart, liver, pancreas, spleen, stomach, skeletal muscle, ovary, kidney, and cultured smooth muscle cells from aorta and uterus. Additional bands of approximately 45 kDa, 58 kDa, and several other larger species were also found among some of the tissues analysed. Bands of lower molecular mass than 28 kDa were never seen, indicating that protein breakdown was not occurring in our lysates. Comparison of lysates made from the same tissues in both rat and mouse showed that the pattern of tissue specific variants was the same in both animals. The sum of the band intensities of all CCN5 variants in each tissue lysate was proportional to the expression level of CCN5 in IHC experiments. These data suggest that CCN5 is modified in vivo by tissue specific post-translational modification(s) in many, if not all, organs and tissues. It is possible that different arrays of post-translational modifications contribute to cell and tissue specific modulation of CCN5 function and signalling.

P4 OESTROGEN REGULATION OF CCN5 EXPRESSION AND FUNCTION

L. Oleson[1], M. Senn[2], J. Jones[2], J. Russo[2], M. Gray[2], J. Castellot[1,2]. [1]*Department of Pharmacology and Experimental Therapeutics and* [2]*Department of Anatomy and Cell Biology, Tufts University School of Medicine, Boston MA, USA*

Epidemiological studies have shown that premenopausal women are much less likely to develop cardiovascular disease than age matched men or postmenopausal women, suggesting that oestrogen may offer women protection against the development of this disease. Arteriosclerosis, the major form of cardiovascular disease, is characterised by hyperproliferation of vascular smooth muscle cells (VSMC). Furthermore, uncontrolled proliferation of uterine smooth muscle cells (UtSMC) is the cause of leiomyomas (fibroids). CCN5 has been shown to inhibit both VSMC and UtSMC proliferation in culture. Although we have shown previously that oestrogen strongly upregulates CCN5 in UtSMC, the effect of oestrogen on CCN5 expression in VSMC has not been explored. Therefore, we examined the effect of oestrogen on CCN5 expression and proliferation in cultured rat VSMC using physiological doses of 17β oestradiol (10^{-10} to 10^{-8}M) over the course of four days. Western blot analysis showed a strong dose dependent induction of CCN5 by oestrogen. Both 28 kDa and 47 kDa CCN5 variants were identified. When we examined the effect of oestrogen on cell proliferation, we found a dose dependent inhibition of VSMC cell number, in agreement with several earlier investigators. The inhibitory action of CCN5 on both VSMC and UtSMC and the ability of oestrogen to induce CCN5 expression in both cell types represents a potentially promising insight into our understanding of the pathological role of CCN5.

P5 ANALYSIS OF DOWNSTREAM REGULATORS OF CCN5 FUNCTION USING GENE MICROARRAY

J. Russo, M. Gray, J. Castellot. *Department of Anatomy and Cell Biology, Tufts University School of Medicine, Boston MA, USA*

Smooth muscle cells (SMC) in the myometrium of the uterus are involved in several uterine pathologies, including leiomyomas (fibroids). The clinical presence of leiomyomas in the general female population is 15–20%, and the prevalence rises to > 60% in black women. To date, the only effective treatment is hysterectomy. The development of less drastic therapeutic alternatives requires a detailed understanding of the cellular and molecular mechanisms regulating both leiomyoma and normal SMC. Earlier work in our laboratory showed that neoplastic SMC isolated from fibroids display no endogenous production of CCN5 protein, whereas SMC derived from the myometrium of the same patients display normal levels of CCN5. In addition, forced expression of CCN5 using an adenoviral system inhibits the proliferation and motility of cultured leiomyoma cells. Importantly, neither the upstream nor downstream regulatory elements of the biological activities of CCN5 have been examined. The objective of the present study is to use gene microarray technology to determine potential candidates for downstream effector molecules in uterine SMC. Using an adenoviral infection system, CCN5 protein production was unregulated in cultured human uterine myometrial cells. RNA extracted from the cells was hybridised to a human gene microarray slide containing 21 000 genes. Three separate slides were analysed and compared; all displayed highly consistent changes. Preliminary analysis indicates that, in total, 505 genes (0.024%) display a threefold or greater change in the presence of increased CCN5. Prominent among the CCN5 regulated genes was a cluster of approximately 30 extracellular matrix associated genes. The CCN5 regulated proteins include collagens, matrix metalloproteinases, integrins, and others. Although specific interpretation of the data requires further analysis, the ability of CCN5 to regulate a relatively large group of extracellular matrix associated genes suggests an important role for this protein class in mediating the mechanism of action of CCN5.

P6 TRANSGENIC APPROACHES TO ANALYSING CCN5 FUNCTION

J. Jones, T. Aprahamian, J. Castellot. *Department of Anatomy and Cell Biology, Tufts University School of Medicine, Boston MA, USA*

CCN5 is a growth arrest specific gene that inhibits vascular smooth muscle cell (VSMC) proliferation. We demonstrated previously that CCN5 expression decreases dramatically during the period of cell proliferation that follows vascular injury. In vitro, growth arrested VSMC strongly express CCN5; expression levels decrease during exponential growth. We hypothesise that CCN5 overexpression during arterial injury will attenuate lesion formation. To test this hypothesis, we are using two approaches. The first examines whether overexpressing CCN5 via an adenoviral vector (Ad-CCN5) following vascular injury protects wild-type mice from neointimal hyperplasia. In this model, a small wire threaded into the external carotid artery completely denudes endothelial cells and induces VSMC proliferation. Medial thickening and VSMC hyperplasia are measured at several time points after carotid wire injury to ascertain the degree of VSMC proliferation and CCN5 expression levels and patterns. Production and characterisation of Ad-CCN5 and control vectors (Ad-green fluorescent protein) is already complete and mouse injury experiments are under way. The second approach uses CCN5 overexpressing transgenic mice. We predict that such mice will exhibit a diminished response to vascular injury when compared with wild-type mice. We are producing transgenic mice that overexpress mouse CCN5 cDNA with a C-terminal HA tag. CCN5 will be expressed under the control of the tissue specific promoter, SM22α (−436/+43). This promoter restricts transgene expression to arterial but not venous or visceral smooth muscle cells. We have deleted a G/C rich repressor in the SM22α promoter that can downregulate transgene expression following vessel injury, thus allowing the promoter to maintain high levels of VSMC transgene expression throughout the period of injury and repair. We also plan to produce mice that conditionally express CCN5 through a tetracycline induced expression system. We are generating two lines of mice: the first will conditionally express the transgene in all tissues under the control of a modified cytomegalovirus or Simian virus 40 promoter, and the second will restrict conditional expression to the arterial wall under the control of the SM22α promoter. The experiments discussed here may be clinically useful in attenuating VSMC hyperproliferation and restenosis after vascular surgery.

P7 INTERNAL RIBOSOME ENTRY SITE (IRES) ACTIVITY IN THE 5′ UTRS OF CCN1, CCN2, AND CCN4

L. M. Dornbach[1], K. M. Lyons[1,2,3]. *Departments of [1]Biological Chemistry and [2]Molecular, Cellular, and Developmental Biology, University of California at Los Angeles, Los Angeles, CA, USA; [3]Department of Orthopedic Surgery, David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, CA, USA*

During normal conditions, the process of protein translation requires binding of ribosomes and translation initiation factors to capped mRNA. However, a variety of cellular conditions, such as hypoxia and apoptosis, inhibit the process of cap dependent translation. Under these conditions, an alternative mechanism of translation must exist for the cell to synthesise essential proteins. A few cellular genes have been found to contain internal ribosome entry sites (IRES) within the 5′ untranslated regions (UTRs), allowing ribosomes to bind.

The 5′ UTRs of ccn1, ccn2, and ccn4 have certain features that are found in the few cellular genes known to contain IRES sites, such as a high GC content and multiple open reading frames. Furthermore, ccn1, ccn2, and ccn4 are all expressed in the growth plate of cartilage, a hypoxic tissue that might be expected to use IRES mediated translation. Therefore, we investigated whether these genes contain functional IRES sites. The presence of a functional IRES site in a 5′ UTR can be tested in vitro with bicistronic constructs containing the 5′ UTR and a firefly luciferase cassette. In transient transfections, we find that the 5′ UTRs of ccn1, ccn2, and ccn4 activate firefly luciferase 50- to 100-fold over basal levels in HeLa cells in both normoxia and hypoxia. The 5′ UTRs of ccn1, ccn2, and ccn4 are also able to activate firefly luciferase at more modest levels in ATDC5, MC3T3-E1, and HUVEC cells, three cell lines that resemble the types of cells found in the growth plate. Our results suggest that the 5′ UTRs of ccn1, ccn2, and ccn4 contain functional IRES sites that may facilitate translation in the growth plate under conditions of hypoxia. These results may also be relevant to the ability of certain proangiogenic CCN genes to promote tumour growth and metastasis.

P8 REDUCED CTGF EXPRESSION LEVEL IN PODOCYTES PREVENTS GLOMERULAR BASEMENT MEMBRANE (GBM) THICKENING IN DIABETIC MICE

P. Roestenberg[1], R. Verheul[1], K. M. Lyons[3], J. A. Joles[2], J. Aten[4], P. W. Mathieson[5], M. Saleem[5], F. A. van Nieuwenhoven[1], R. Goldschmeding[1]. *Departments of [1]Pathology and [2]Nephrology and Hypertension, UMCU, Utrecht, Netherlands; [3]Department of Orthopedic Surgery, UCLA, Los Angeles, CA, USA; [4]Department of Pathology, AMC, Amsterdam, Netherlands; [5]Academic Renal Unit, University of Bristol, Bristol, UK*

Connective tissue growth factor (CTGF) is a 36–38 kDa secreted protein that is strongly upregulated in fibrotic disorders, and may be a pathogenic factor in the development of diabetic nephropathy (DN). In the present study, we investigated the localisation and expression levels of CTGF in relation to ECM accumulation in streptozotocin (STZ) induced diabetic CTGF+/− and wild-type (CTGF+/+) mice. CTGF+/− mice (BalbC/129SV) were crossbred with wild-type C57Bl6/J mice. Diabetes mellitus (DM) was induced by means of STZ (intraperitoneally, 200 mg/kg) in the adult female F1 generation. Female littermates served as controls. At nine weeks of DM, all mice were uninephrectomised to accelerate the progression of DN. Mice were sacrificed after 17 weeks of DM. The localisation of CTGF was assessed by in situ hybridisation (ISH). CTGF and extracellular matrix (ECM) protein and mRNA levels in the renal cortex were determined by quantitative polymerase chain reaction. Glomerular basement membrane (GBM) and tubular basement membrane (TBM) thicknesses were measured by transmission electron microscopy and mesangial matrix accumulation was quantified (periodic acid Schiff staining).

Q-PCR showed that CTGF gene expression levels were threefold upregulated in renal cortex of DM+/+ mice compared with controls. In DM+/− mice, CTGF mRNA was also increased, but remained two times lower than in DM+/+ mice. In contrast to DM+/+ mice (17% increase), GBM thickness in DM+/− mice was not increased compared with normoglycaemic controls. No differences in TBM thickness were seen between the different groups. ISH revealed that CTGF expression is mainly localised in podocytes. In addition, in vitro studies showed that fibronectin expression in human podocytes was induced dose dependently by CTGF. However, albuminuria, mesangial matrix, blood urea nitrogen, and mRNA levels of fibronectin and collagen IV in total renal cortex were all equally increased in both DM+/+ and DM+/− mice compared with normoglycaemic controls.

In this model of type 1 DM, BM thickening in the renal cortex is limited to the GBM—the area of CTGF overexpression. These results indicate that CTGF expression in podocytes is involved in DM related GBM thickening, but does not correlate with albuminuria, renal function, or ECM increase of renal cortex.

P9 CONNECTIVE TISSUE GROWTH FACTOR AND ITS ROLE IN LUNG ADENOCARCINOMA INVASION AND METASTASIS

C-Y. Yang[1,2,3,4], C-C. Chang[2], C-N. Chen[1,3], F-J. Hsieh[1,5], P-H. Lee[1,3], K-J. Chang[1,3], P-C. Yang[6], M-L. Kuo[1,2]. *[1]Angiogenesis Research Centre and [2]Laboratory of Molecular and Cellular Toxicology, Institute of Toxicology, College of Medicine, National Taiwan University, Taiwan; Departments of [3]Surgery, [4]Traumatology, [5]Obstetrics and Gynaecology, [6]Internal Medicine, National Taiwan University Hospital, Taipei, Taiwan, ROC*

Background/Aims: Tumour invasion and metastasis are the cause of most deaths in patients with cancer. Connective tissue growth factor (CTGF), a secreted protein that binds to integrins, modulates the invasive behaviour of certain human cancer cells, but few mechanistic details are known. The roles of CTGF and collapsin response mediator protein 1 (CRMP-1) in metastasis and invasion of human lung adenocarcinoma were investigated.

Methods: Vector control transfected cells were compared with corresponding CTGF gene transfected cells. Invasive activity was measured with a modified Boyden chamber assay, and metastatic activity was measured in an animal model. CTGF deletion mutants, CTGF and CRMP-1 antisense oligonucleotides, and anti-integrin and anti-CRMP-1 antibodies were used to investigate the functional relation between CTGF and CRMP-1. Expression of CTGF protein in 78 lung adenocarcinoma specimens was investigated immunohistochemically. All statistical tests were two sided.

Results: Invasive (both $p < 0.001$) and metastatic ($p < 0.001$ and $p = 0.003$, respectively) activities were lower in cells that overexpress CTGF than in vector control cells. Expression of CRMP-1 was higher in CTGF transfected clones than in vector control cells, and its level decreased after cells were treated with anti-integrin $\alpha_v\beta_3$ and $\alpha_v\beta_5$ antibodies. Reduced levels of CRMP-1 protein after the transfection of CRMP-1 specific antisense oligonucleotides, but not sense oligonucleotides, increased the invasiveness of CTGF transfected cells (mean numbers of invasive CTGF transfected cells treated with 20 μM CRMP-1 specific sense and antisense oligonucleotides were 327 and 516 cells, respectively; difference, 189; 95% confidence interval (CI), 156 to 221; $p < 0.001$). The CT module of CTGF was the region primarily responsible for the increased expression of CRMP-1 and the inhibition of invasion (mean numbers of invasive cells expressing full length CTGF and CT module deleted mutant were 148 and 385 cells, respectively; difference, 237; 95% CI, 208 to 266; $p < 0.001$). Reduced expression of CTGF in lung cancer specimens was significantly associated with the risk of more advanced stage disease (stages III and IV *v* stages I and II; $p = 0.001$), lymph node metastasis ($p = 0.014$), and shorter survival (median survival with high and low levels of CTGF, 66.7 and 18.2 months, respectively; difference, 48.5; 95% CI, 33.5 to 63.5; $p = 0.02$).

Conclusion: CTGF inhibits metastasis and invasion of human lung adenocarcinoma by a CRMP-1 dependent mechanism.

P10 GLOBAL GENE EXPRESSION ANALYSIS REVEALS A ROLE FOR THE RHO–ROCK PATHWAY IN INTESTINAL SMOOTH MUSCLE CELL FIBROGENIC DIFFERENTIATION

M. C. Vozenin-Brotons[1,2], C. Bourgier[1,2], V. Haydont[1,2], F. Milliat[1,2], A. François[1,2], V. Holler[2], P. Lasser[4], J. Bourhis[1,3], D. Mathé[2]. *[1]UPRES EA 27-10, Radiosensibilité des Tumeurs et Tissus Sains, IGR/IRSN Villejuif, France; [2]Laboratoire d'étude des Pathologies Radio-induites, SRBE/DRPH, IRSN, Fontenay-aux-Roses, France; [3]Département de Radiothérapie, IGR, Villejuif, France; [4]Département de Chirurgie, IGR, Villejuif, France*

Background/Aims: Radiation enteritis is associated with activation of intestinal smooth muscle cells and increased CTGF expression.[1] cDNA array analysis revealed alteration of the genes encoding the Rho family of proteins.[2] Because Rho proteins are involved in the regulation of stress fibre formation and may regulate CTGF expression, alteration of the Rho pathway may be involved in the maintenance of radiation induced smooth muscle cell fibrogenic differentiation.

Methods: Radiation enteritis and normal ileum biopsies were dissected and muscularis layers were enzymatically digested to establish primary cultures of smooth muscle cells. Cytoskeleton structure and composition and CTGF and type I collagen secretion were studied. The involvement of the Rho–ROCK pathway in the fibrosis activated smooth muscle cells was assessed using Y-27632, a specific inhibitor of Rho kinase.

Results: Intestinal smooth muscle cells retained their fibrogenic differentiation in vitro and exhibited a typical cytoskeletal network. CTGF and pro-collagen I secretion was enhanced in cells derived from radiation enteritis compared with their normal counterparts and was concomitant to alteration of the Rho pathway. Specific inhibition of Rho kinase with Y-27632 induced a simultaneous decrease in the number of actin stress fibres, and in α-smooth muscle actin and heat shock protein 27 protein levels. Y-27632 also decreased CTGF concentrations, probably via nuclear factor κB inhibition and the type I collagen gene.

Conclusions: The present study suggests that the Rho–ROCK pathway is involved in the control of the actin network and in the regulation of CTGF expression associated with radiation induced fibrogenic differentiation in intestinal smooth muscle cells. Furthermore, inhibition of the Rho/ROCK pathway seemed to reverse radiation induced fibrogenic differentiation in vitro, suggesting that specific inhibition of Rho kinase may be a promising approach for the development of antifibrotic treatments.

1. **Vozenin-Brotons MC**, *et al. Int J Radiat Oncol Biol Phys* 2003;**56**:561–72.
2. **Vozenin-Brotons MC**, *et al. Radiat Res* 2004;**3**:299–311.

P11 MAPPING DIFFERENTIAL MECHANISMS OF CCN2 (CTGF) GENE REGULATION: INDUCTION OF CCN2 BY TGFβ AND THROMBIN BY DIVERGENT SIGNALLING PATHWAYS

A. M. Holmes[1], P. Garcia[1], M. Sabetkar[1], X. Shi-Wen[1], A. Leask[1], M. Ponticos[1], R. C. Chambers[2], C. M. Black[1], D. J. Abraham[1]. *[1]Centre for Rheumatology, Department of Medicine, Royal Free and University College Medical School, Rowland Hill Street, London NW3 2PF, UK; [2]Centre of Cardiopulmonary Biochemistry and Respiratory Medicine, Royal Free and University College Medical School, UCL, Rayne Institute, 5 University Street, London WC1E 6JJ, UK*

CCN2 (CTGF) has been shown to be involved in normal tissue repair including the wound healing process following injury to the dermis. In vitro, CCN2 elicits several biological responses that are of potential importance in the healing response, including the stimulation of cell proliferation, cell adhesion, chemotaxis, and angiogenesis, in addition to the production of extracellular matrix (ECM) components. Thus, CCN2 has been proposed as a key downstream effector of factors promoting wound healing, including transforming growth factor β (TGFβ) and thrombin. CTGF has also been found to be overexpressed in numerous fibrotic conditions including artheriosclerosis, renal diseases, and systemic sclerosis, the pathology of which exhibits features of excessive wound healing and tissue repair. This study investigates the transcriptional and cell signalling mechanism(s) by which thrombin and TGFβ, key modulators of the ECM and of fibroblast biology, induce CCN2 expression in dermal fibroblasts.

Using real time polymerase chain reaction and western blot analysis we found that thrombin and TGFβ were able to induce CCN2 mRNA and protein expression, with TGFβ being the most potent. Using the previously cloned CTGF promoter reporter construct containing 800 bp downstream of the TATA box we found that TGFβ but not thrombin was able to induce reporter gene expression. Transfection of the deletion series showed that TGFβ induced CCN2 between −166 and −244 bp downstream of the TATA box. By generating CCN2 promoter reporter mutations in a putative SMAD binding site and the formally identified TGFβ response element (BCE-1) binding site within the −166 and −244 bp region in the context of a 800 bp CCN2 reporter construct, we confirmed that the SMAD site but not the BCE-1 site was essential for TGFβ induction.

To investigate further the mechanism of induction and delineate the specific regions responsive to the profibrotic factor thrombin we cloned 5 kb of the human CCN2 promoter linked reporter gene. To our surprise, we found that the thrombin response element in the CCN2 promoters resided between nucleotides −5000 and −800 bp.

In conclusion, the profibrotic factors thrombin and TGFβ act to induce CCN2 gene activity via discrete elements in the promoter, suggesting that these factors may cooperate to induce increased expression of CCN2 during wound healing responses in vivo.

P12 DIFFERENTIAL GENE EXPRESSION OF CCN FAMILY MEMBERS IN RETINAS OF VEGF INJECTED RAT EYES

I. Klaassen, E. J. Kuiper, J. M. Hughes, R. O. Schlingemann. *Department of Ophthalmology, Academic Medical Centre, Amsterdam, The Netherlands*

Proliferative diabetic retinopathy (DR) and exudative age related macular degeneration (AMD) are the leading causes of blindness in Western countries. The progression of both diseases is complicated by neovascularisation and fibrosis, which eventually lead to irreversible loss of vision. The major factor responsible for neovascularisation was

identified as vascular endothelial growth factor A (VEGF). The precise mechanism of action of VEGF has not been elucidated to date, but it is thought to act in concert with many other cytokines. A recently discovered growth factor, which also plays a role in extracellular matrix production and fibrosis, is connective tissue growth factor (CTGF). In vitro studies have shown that VEGF can induce CTGF transcription in retinal endothelial cells and pericytes. To study VEGF induced expression in vivo, we investigated mRNA expression patterns of CTGF in retinas of VEGF injected rat eyes. In addition, we also investigated mRNA expression of other CCN family members and extracellular matrix components. By means of quantitative reverse transcription polymerase chain reaction we demonstrated a significant induction of CTGF and CYR61 24 hours after VEGF injection. Forty eight hours after injection, CTGF transcription levels were on average higher, although no longer significantly, than in control retinas and CYR61 expression was back to normal. With regard to the remaining CCN family members, expression levels were unchanged for CCN4, whereas CCN3, CCN5, and CCN6 expression was undetectable. Fibronectin and transforming growth factor β2 (TGFβ2) expression was raised significantly 24 hours after injection and decreased 48 hours after injection to levels that were not significantly different from controls. This was in contrast to expression levels of collagen type IV, TGFβ1, and tissue inhibitor of metalloproteinase 2, which remained comparable to control levels at both time points. Taken together, VEGF injection in rat eyes induces upregulation of retinal CTGF, CYR61, and TGFβ2 genes and selected extracellular matrix genes. In vivo regulation of these genes by VEGF may be a crucial mechanism underlying fibrosis and neovascularisation in the progression of DR and AMD.

P13 COMPARATIVE ANALYSIS OF SPATIOTEMPORAL EXPRESSION OF CCN1 (CEF10) AND CCN3 (NOV) GENES IN CHICK EMBRYO

B. Bessette[1], B. Perbal[2], C. Li[2], C. Ayer Le Lièvre[1], M. Mathonnet[1]. [1]*EA 3842, Homéostasie Cellulaire et Pathologies, Faculté de Médecine, 2 Rue du Docteur Marcland, 87025 Limoges Cedex, France;* [2]*Laboratoire d'oncologie Virale et Moléculaire, UFR de Biochimie, Université Paris 7-D. Diderot, 2 Place Jussieu, 75005 Paris, France*

Control of cell proliferation requires the interplay of many signalling factors. All the genes of the CCN family share a common multimodular organisation encoding both positive and negative regulators of cell growth. The two first members (CCN1 and CCN2) of this gene family are known to be immediate–early genes. Comparing the expression of CCN1 (Cef10) and CCN3 (Nov) at different developmental stages in chick embryos (E7 to E8) should help to determine their role in morphogenesis and differentiation during embryonic development and their sequential activation. In situ hybridisation has been used to investigate the spatiotemporal pattern of CCN1 (Cef10) and CCN3 (Nov) gene expression in chick embryo limbs. These genes appear to be expressed sequential during chick limb formation.

P14 CONNECTIVE TISSUE GROWTH FACTOR CORRELATES WITH TISSUE INHIBITOR OF MATRIX METALLOPROTEINASE-1 IN A BABOON MODEL OF DIABETIC NEPHROPATHY

S. E. Thomson[1,2], S. V. McLennan[3], S. Heffernan[2], A. Hennessy[1,2], D. K. Yue[1,3], S. M. Twigg[1,3]. [1]*Discipline of Medicine, University of Sydney, Sydney, Australia; Departments of* [2]*Renal Medicine and* [3]*Endocrinology, Royal Prince Alfred Hospital, Sydney, NSW, 2006 Australia*

Diabetic nephropathy is characterised by the accumulation of extracellular matrix, glomerulosclerosis, and tubulointerstitial fibrosis. Previous studies have shown increased connective tissue growth factor (CTGF) staining in glomeruli and proximal tubules in rodent and human diabetes. We have recently shown that CTGF upregulates the tissue inhibitor of matrix metalloproteinase type 1 (TIMP-1) in mesangial cells, which then results in inhibition of matrix degradation, suggesting that in diabetes CTGF may contribute to matrix accumulation through TIMP-1. In this work, we examined renal biopsies from our colony of diabetic baboons to determine whether CTGF is related to TIMP-1 and matrix expansion. Male diabetic (average duration, 60.2 months; age, 7.4 years; weight, 16.7 kg; glycated haemoglobin (HbA1c), 9.2%; microalbuminuria, 7.3 μg/min), and control baboons (average duration, 60.2 months; age, 7.2 years; weight 23.4 kg; HbA1c, 4.6%; microalbuminuria, < 3 μg/min) were studied. Renal biopsies were examined histologically for CTGF, TIMP-1, and fibronectin staining, and for changes in mesangial volume. Glomerular and proximal tubular CTGF, TIMP-1, and fibronectin levels were graded as 0 (no staining) to 3 (intense staining) by two observers blinded to tissue source. Mesangial volume was also reported by a pathologist. Averaged scores were grouped into staining scores $\leqslant 1$ or > 1.

CTGF in glomeruli and tubules, TIMP-1 in glomeruli, and mesangial volume were significantly increased in diabetic animals. After excluding one TIMP-1 control outlier, the staining score for CTGF positively correlated with the staining score for TIMP-1 ($r = 0.51$; $p = 0.037$). These semiquantitative data are consistent with upregulation of CTGF in this primate model of diabetic nephropathy, leading to increased TIMP-1 and consequent mesangial matrix expansion.

Supported by Juvenile Diabetes Research Foundation International, DART Millenium Grant and Rebecca Cooper Foundation.

Abstract P14

	CTGF (% >1)		TIMP-1 (% >1)		F (% >1)		MV (% >1)
	T	G	T	G	T	G	G
C (n=7)	28.6	14.3	14.3	14.3	79.5	42.9	29.0
D (n=9)	76.8*	76.8*	22.3	66.7*	80.0	80.0	66.0*

*p<0.05 compared with respective control.
C, control baboons; CTFG, connective tissue growth factor; D, diabetic baboons; F, fibronectin; G, glomeruli; MV, mesangial volume; T, tubules; TIMP-1, tissue inhibitor of matrix metalloproteinase type 1.

P15 DIFFERENTIAL REGULATORY EFFECTS OF SOLUBLE AND MATRIX BOUND ADVANCED GLYCATION END PRODUCTS ON PROFIBROTIC CYTOKINES

X. Wang[1], S. V. McLennan[2], S. M. Twigg[1,2]. [1]*Discipline of Medicine, University of Sydney, Sydney, Australia;* [2]*Department of Endocrinology, Royal Prince Alfred Hospital, Sydney, NSW, 2006 Australia*

Advanced glycation end products (AGEs) accumulate in tissues and are increased in biological fluids of diabetic subjects, and may contribute to end organ complications through upregulation of cytokines. We have previously shown that connective tissue growth factor (CTGF), also known as CCN2, is increased in mesenchymal cells by soluble AGEs. We now tested whether AGEs bound to extracellular matrix increase CTGF, and compared these results with the regulation of transforming growth factor β1 (TGFβ1).

Preformed mesangium matrix in solid phase or bovine serum albumin (BSA) in solution were glycated by incubation in 500mM ribose at 37°C for 10 days. As assessed by fluorescence, glycation was increased threefold in the matrix and fivefold in the soluble BSA. In some experiments, the glycation inhibitor aminoguanidine was coincubated with the ribose and matrix or BSA, as a negative control. Primary human fetal mesangial cells were cultured on glycated matrix, or treated in

Abstract P15

	Extracellular matrix (solid phase)			Albumin (soluble phase)		
mRNA species	Matrix control	Glycated matrix	AG matrix	BSA control	AGE-BSA	AG-BSA
CTGF	100% (17%)	148% (10%)*	84.4 (12%)	100% (7.5%)	136% (12%)*	90.8% (3.2%)
TGFβ1	100% (17%)	62.8% (6.2%)*	94.2% (14%%)	100% (19%)	276% (8.4%)*	110% (2.9%)

Values are mean (SD).
*p<0.05 *v* control.
AG, aminoguanidine; AGE, advanced glycation end products; BSA, bovine serum albumin; CTGF, connective tissue growth factor; TGFβ1, transforming growth factor β1.

Abstract P16

Animal group	Body weight (g)	ITT plasma glucose mean fall AUC (mmol/l)	CTGF intact protein (% of control)	CTGF fragment to intact ratio (% of control)	CTGF mRNA (% of control)
PVG/c +vehicle	374 (5.4)	1.67 (0.34)	100	100	100
PVG/c +pio	373 (5.0)	2.41 (0.52)	194 (48)*	86 (20.1)	215 (36)*
PEPCK +vehicle	411 (12.0)†	1.47 (0.25)	81 (31)	146 (20.0)†	265 (28)*
PEPCK +pio	450 (5.4)†‡	2.51 (0.48)‡	158 (3)‡	68 (12.4)†‡	193 (25)*†

Data are mean (SEM).
*p<0.05 *v* PVG/c +vehicle group; †p<0.05 *v* each PVG/c group; ‡p<0.05 *v* PEPCK +vehicle; all analysis is by multiple ANOVA.
AUC, area under the curve; CTGF, connective tissue growth factor; ITT, insulin tolerance test; PEPCK, rats overexpressing phosphoenolpyruvate carboxykinase; pio, pioglitazone; PVG/c, control rats.

monolayer with soluble AGEs, compared with respective controls. After 48 hours, total RNA was isolated. CTGF and TGFβ1 values were measured by quantitative real time reverse transcription polymerase chain reaction, corrected for the total amount of RNA.

CTGF mRNA was increased by both glycated matrix and soluble AGEs. This increase was prevented by aminoguanidine. TGFβ1 mRNA was also increased by soluble AGEs. However, in contrast to CTGF, TGFβ1 was downregulated by glycated matrix. These results indicate that these cytokines are differentially regulated by matrix bound AGEs, and CTGF is probably upregulated by glycated matrix, independently of TGFβ1. We propose that CTGF induction, rather than TGFβ1, is the primary mechanism by which glycated matrix causes matrix accumulation.

Supported by National Health and Medical Research Council of Australia.

P16 REGULATION OF CONNECTIVE TISSUE GROWTH FACTOR IN ADIPOSE TISSUE BY THE METABOLIC SYNDROME AND THIAZOLIDENEDIONE

L. Lo[1], X. Wang[1], P. F. Williams[1], J. Tan[1], F. V. McDougall[1], S. S. Y. Chan[3], J. Proiettto[4], J. D. Zajac[4], S. V. McLennan[1,2], S. M. Twigg[1,2]. *[1]Department of Endocrinology, Royal Prince Alfred Hospital, Sydney, Australia; [2]Discipline of Medicine, [3]Kolling Institute of Medical Research, University of Sydney, Sydney, NSW 2006 Australia; [4]Department of Medicine, Repatriation Hospital, Heidelberg, Australia*

There is no information on the multifunctional protein, connective tissue growth factor (CTGF), also known as ccn2, in adipose tissue or in the metabolic syndrome (MetS). Because CTGF is potently regulated by transforming growth factor β (TGFβ) in many cell types and because TGFβ regulates adipose tissue differentiation, we studied the regulation of CTGF in adipose tissue.

A genetically induced rodent model of MetS involving overexpression of the phosphoenolpyruvate carboxykinase (PEPCK) gene was studied. Wild-type, age matched (4 months) male PVG/c hooded Wistar rats were used as controls. Animals (n = 5–6/group) were treated with vehicle alone or the insulin sensitising agent, pioglitazone (pio), orally 20 mg/kg body weight/day, for 28 days. Intravenous insulin tolerance tests (ITTs) were then performed and tissues were isolated at termination. Epididymal fat data are shown.

PEPCK animals were heavier than controls, and pio improved their insulin sensitivity and markers of fat cell differentiation (not shown). Intact CTGF protein in tissue lysates, as assessed by western analysis, was upregulated by pio. A pronounced increase in the relative amount of a 20 kDa C-terminal CTGF fragment in MetS was downregulated by pio. CTGF mRNA measured by real time reverse transcription polymerase chain reaction was increased by MetS and was upregulated by pio. These data suggest that CTGF is proteolysed in epididymal fat by MetS, and the increases in CTGF mRNA in MetS may be counter-regulatory. Pio upregulates CTGF protein in epididymal fat. Further studies are required to determine the significance of these changes in central body fat.

Supported by a competitive grant from Eli Lilly.