

## Correspondence

### Formic acid decalcification of bone marrow trephines degrades DNA: alternative use of EDTA allows the amplification and sequencing of relatively long PCR products

We recently reported a new method for the extraction of DNA from paraffin wax embedded bone marrow trephine biopsies.<sup>1</sup> The DNA extracted from EDTA decalcified bone marrow trephine biopsies using this method was sufficiently intact to allow the amplification and sequencing of relatively long polymerase chain reaction (PCR) products, including the 600 bp t(11;14) MTCA PCR product. A shorter 294 bp PCR product could only be amplified from six of 10 formic acid decalcified bone marrow trephine biopsies reported in a previous study by Provan *et al.*<sup>2</sup> These findings suggested a correlation between DNA degradation and formic acid decalcification, but required a comparative study for confirmation.

We have subsequently extracted DNA from 11 formic acid decalcified bone marrow trephine biopsies using our method and determined the quality of DNA using agarose gel electrophoresis and PCR analysis, as in our initial study.

The mean DNA yield from the formic acid decalcified blocks was twice that of the EDTA decalcified samples: 9.4 µg and 4.3 µg, respectively. This reflected the fact that the formic acid blocks contained approximately twice as much bone marrow trephine biopsy material as a result of the differences in practice between the two centres involved in the study (Exeter, EDTA decalcification; Southampton, formic acid decalcification). However, when the formic acid decalcified DNA samples were analysed by agarose gel electrophoresis, no high molecular weight DNA was detected; only a smear of degraded DNA was seen. In contrast, analysis of the EDTA decalcified bone marrow trephine biopsy DNA samples showed DNA ranging from 5 to 21 kb in length (fig 1).

It was not possible to amplify the 147 bp factor V PCR product from three of the formic acid decalcified DNA samples. The remaining eight samples generated very weak products compared with control DNA extracted from peripheral blood lymphocytes. This short PCR product was previously amplified successfully from all eight EDTA decalcified bone marrow trephine biopsy DNA samples.

The 482 bp BRCA 1 exon 11B product was only amplified successfully from one of the 11 formic acid decalcified samples. The intensity of the band seen on agarose gel electrophoresis was very weak compared with the control. Previously, all of our EDTA decalcified bone marrow trephine biopsy DNA samples were amplified successfully to generate this product.

Three formic acid decalcified samples yielded BRCA 1 exon 11A products (643 bp). However, the intensity of these products was so weak compared with the control that they were barely visible in the agarose gel. This relatively long PCR product had been amplified successfully using all EDTA decalcified bone marrow trephine biopsy DNA samples; five bands were of a similar intensity to the positive control, two were relatively weak, and one had to be diluted 1/20 to generate a band.<sup>1</sup>

This comparative study strongly suggests that formic acid decalcification of bone marrow trephine biopsies causes DNA degradation, rendering specimens decalcified by this method unsuitable for use as a source of archival DNA.

Consequently, in view of the increased requirement for the use of molecular techniques in the diagnosis and monitoring of patients with lymphoma and leukaemia, the use of formic acid as a bone marrow trephine biopsy decalcifying agent should be reviewed. Decalcification with EDTA has been used routinely in the histopathology department at the Royal Devon and Exeter NHS Trust for several years and, despite the minor delay involved in tissue processing, causes no impairment of the quality of immunohistochemical and tinctoral staining in bone marrow trephine biopsies compared with formic acid decalcification. Directly as a result of the outcome of this comparative study, South-

ampton University Hospitals NHS Trust has now converted from the use of formic acid to EDTA for decalcification of BMT.

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1 Wickham CL, Boyce M, Joyner MV, *et al.* Amplification of PCR products in excess of 600 base pairs using DNA extracted from decalcified, paraffin wax embedded bone marrow trephine biopsies. *J Clin Pathol: Mol Pathol* 2000;53:19-23.

2 Provan AB, Hodges E, Smith AG, *et al.* Use of paraffin wax embedded bone marrow trephine biopsy specimens as a source of archival DNA. *J Clin Pathol* 1992;45:763-5.

## Book reviews

**Therapeutic Interventions in the Complement System.** Lambris JD, Holers VM, eds. (£90.00.) Humana Press, 2000. ISBN 0 896 03587 5

The title misled me at first. At last, how to treat my complement deficient patients with more than antibiotics and vaccination. Nope, the back cover hyperbole quickly dashed that thought. What is presented here is a topical review of ways to inhibit complement and its inflammatory role in many diseases.

The essential role of complement activation is being defined in an increasing number of human diseases. This book details the complement system itself and the search for synthetic or natural inhibitors of the inflammatory pathways of complement. The first chapter gives a concise overview of the complement system and the role of physiological inhibitors. It also summarises several diseases in which turning off complement could be beneficial—for example, ischaemia/reperfusion injury, autoimmune renal disease, and transplantation. It is also the only place in the book where the possible side effects of such treatment are mentioned in any detail. Because the bulk of *in vivo* research has been performed in animal models, the effects on immunity to infection and immune complex mediated disease in humans needs very careful consideration in planning clinical trials, but this subject receives little attention.

Subsequent chapters are consistently structured, highly detailed accounts of different areas of the complement system. The chapters do not divide up into the usual "alternative", "classical", and "lectin" pathways (although the membrane attack pathway is dealt with in its entirety), mimicking the layout in text books, but rather group components with similar physiological roles. Each chapter goes on to explain the rationale behind developing inhibitors relevant to its own part of the pathway or group

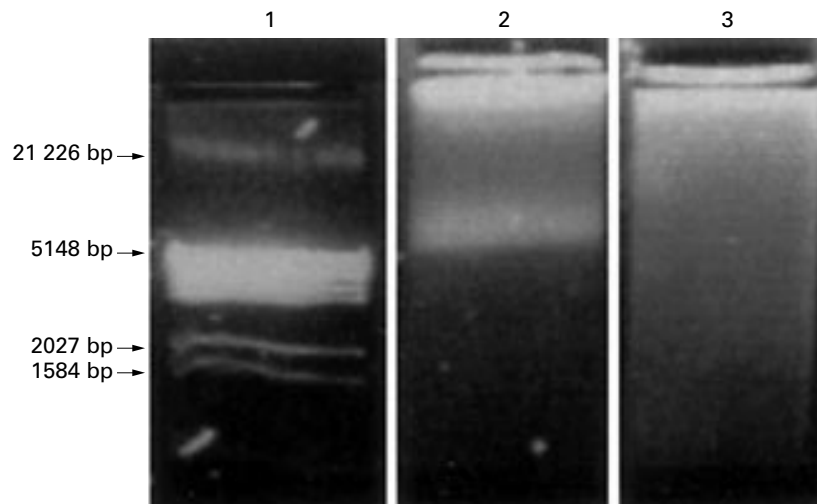


Figure 1 Agarose gel electrophoresis of DNA extracted from EDTA and formic acid decalcified bone marrow trephine biopsies. Lane 1, DNA size standard; lane 2, DNA extracted from an EDTA decalcified bone marrow trephine biopsy; lane 3, DNA extracted from a formic acid decalcified bone marrow trephine biopsy.

of components, how candidate agents are being developed, the use of the agents in animal models, and summaries of any clinical trials up to 1999. The only chapter that meanders a bit is that covering CR3 and CR4 because little bits of therapeutics are mixed in with the structure/function background.

The CR3/CR4 chapter is, however, deserving of a positive comment, because it is the only one that covers enhancing the anti-tumour role of complement. Considering that tumour expressed complement receptors play a major role in tumour escape from host defences, more could have been included. This subject, on the other hand, goes against the thrust of the rest of the book; so why do we get an excellent section on only one aspect of this field of work?

Although the basic activation pathways are described in each chapter, the emphasis varies and there is surprisingly little repetition of fine detail between chapters, showing that the editors have done their job well. Cross referencing within the book is limited and pertinent. The chapters that contain a few lines of summary or conclusions at the end get an extra gold star. I find this very useful in books of this calibre because some areas contain more detail than is needed by an individual reader (yes, I skipped bits!), and the summary lets you know if anything crucial has been overlooked.

The back cover seems to emphasise the coverage of "the new ELISA assays" for assaying complement products. The chapter entitled "Evaluation of complement inhibitors" details (you get a full methodology) the use of haemolytic assays for investigating the effect of putative inhibitors on individual components. It also covers the pros and cons of ELISAs, but anyone looking for details of ELISA methods will be disappointed.

In summary, there is excellent coverage of the structure and function of complement, broken down into slightly novel but rational areas. Details of how and why new therapeutic agents are being developed is comprehensive, as is the use of animal model studies. Clinical information is necessarily scant, and speculation about future developments fills up the gaps left by the book's title. I see no point in trying to decide specifically whom this book is aimed at because the contents will benefit basic scientists and a wide range of clinicians alike.

J NORTH

**The Molecular Basis of Cell Cycle and Growth Control.** Stein GS, Baserga R, Giordano A, *et al.*, eds. (£45.50.) Wiley, 1998. ISBN 0 471 15706 6.

In recent years, there has been an explosion in the number of publications about the mechanisms that control the cell cycle and how their

deregulation can lead to cellular atypia and potentially carcinogenesis. It has become increasingly hard to find review articles that are both up to date and that look at the cell cycle in its entirety. This book, we are delighted to say, attains these criteria. While it starts at the beginning of the study of the cell cycle and attributes important findings to leading investigators, it takes the reader on a journey through the controlling mechanisms of the cell cycle, gradually increasing the detail and amount of information in this very complex subject. Each chapter is written in such a way that it stands alone, providing a rounded review of the topic in question, and yet the chapters also roll together building upon each other.

The first few chapters are devoted to the actions that take place in the different phases of the cell cycle and how these stages link to each other as the concentrations of the associated proteins rise and fall. PL Puri *et al* provide a comprehensive overview of the molecules involved in the cell cycle and how these interact to regulate its progression. Thankfully, they also differentiate between the nomenclature used for genes and proteins associated with the yeast cell cycle and those used for mammalian cells, an area that often causes confusion and unfortunately leads to the erroneous interchange of the two sets of molecules. G Prem Veer Reddy and later Greenfield Sluder *et al* expand upon the mechanism of action and regulation of DNA synthesis and mitosis, respectively; areas that are often glossed over in cell cycle reviews. Gary Stein *et al* elaborate on the transcriptional control of gene expression as the cell traverses from one phase to another and, in particular, they describe how this is used to ensure cell fidelity at the multiple checkpoints through the cycle. This is followed by a lengthy article by David Denhardt, who discusses the reasons why a cell either does or does not proliferate, the effect of exogenous and endogenous stimuli, and the cascade of events that occurs from the initial stimulus to the cell dividing.

The latter part of the book changes its emphasis slightly and looks at the ultimate outcome for a cell: differentiation or death. M Cristina Cardoso and Heinrich Leonhardt highlight the information currently available about the often forgotten act of terminal differentiation, something which should not of course be confused with cell quiescence. They continue to discuss the mechanisms involved in the decision of a cell to apoptose and provide evidence of the dual role that some molecules play in proliferation, differentiation, and apoptosis. Their final contribution is to provide an excellent review of DNA methylation; the current understanding and its role in carcinogenesis.

Another topic that has often led to confusion is cell senescence: how this differs from terminal differentiation and its relation to apoptosis. These concepts are clarified by Judith Campisi, who discusses the need for a finite cell life span and how some cells can bypass these protective mechanisms and become immortalised.

At first glance the final chapter by Bruno Calabretta and Tomasz Skorski does not appear to fit into the theme of the book. However, they use a chronic myeloid leukaemia model as an example of how genes and oncogenes associated with the transformation and maintenance of this disease can be targeted using antisense DNA. Hence, they show that the in depth study of the mechanisms controlling the cell cycle, and how these are altered in tumour cells, is not only of general interest but has great potential in the treatment of malignant disease.

The book makes very good use of diagrams to clarify the text; in particular, there are several colour plates in the middle of the book of both photomicrographs and diagrams, which are replicated in black and white at the relevant point in the text. As with any multi-author book, there is repetition of information, particularly because the cell cycle is introduced at the beginning of each chapter. This does not detract from the book, in fact it makes it easier when reading about one particular aspect of the cell cycle, and if needs be one can always skip over these introductions.

This is a well written and constructed book on factors that influence cell cycle and growth. It is extremely well referenced and we would recommend it to any one with an interest in the cell cycle.

C E GILLET  
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## Books received

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**Cell Encapsulation Technology and Therapeutics.** Kuhtrieber WM, Lanza RP, Chick WL. Birkhauser, 1999. ISBN 3 7643 4010 X.

**Infectious Disease Pathology: Clinical Cases.** Woods GL, Walker D, Winn W, *et al.* Butterworth Heinemann, 1999. ISBN 0 7506 9673 7.

**Gene Quantification.** Ferre F, ed. Birkhauser, 1998. ISBN 3 7643 3945 4.

# The First International Workshop on CCN Proteins

## Posters

### P1 DIFFERENTIAL EXPRESSION OF NOVH PROTO-ONCOGENE IN HUMAN PROSTATE CELL LINES AND TISSUES

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The novH gene is the human homologue of the chicken nov proto-oncogene, which is overexpressed in avian nephroblastomas. The NOVH protein is a member of the CCN family of proteins likely to play a role in cell growth regulation. We have investigated novH expression at RNA and protein levels in prostate epithelial cell lines derived from normal tissues (SV40 large T antigen immortalised PNT1A and PNT1B) and metastatic tumours (LNCaP, DU-145, and PC-3), as well as in patients with benign prostatic hyperplasia (BPH) and prostatic adenocarcinoma. While LNCaP, DU-145, and PC-3 metastatic cells expressed high levels of novH RNA, PNT1A and PNT1B cells expressed low to undetectable levels of novH RNA. Overexpression of novH was detected in PC3 which induce tumour formation and metastasis in nude mice.

The BPH samples expressed variable levels of novH RNA and showed a strong positivity of acinar epithelial cells after immunohistochemical staining.

Prostatic adenocarcinomas showed granular, cytoplasmic immunoreactivity similar to that seen in normal and hyperplastic acinar cells. Some glands showed diffuse staining of their contents or dense, ring-like staining related to gland lumina. Apocrine secretion of NOVH by prostatic cells accounts for the positive reaction of the luminal contents. Although the function of NOVH in the prostatic fluids remains unclear, measuring NOVH levels in seminal fluid might be useful as a marker of prostate diseases. Using immunocytochemistry, all cell lines, except PNT1A and PNT1B, were shown to contain NOVH protein.

Our present results lead us to speculate that NOVH might have a role in the tumorigenic potential of the prostate tumor cells and their ability to metastasise in nude mice.

### P2 THE CYR61 AND THE NON-MUSCLE MYOSIN HEAVY CHAIN-B ARE THE TWO GENES WHOSE EXPRESSIONS ARE SUPPRESSED IN RAT 6 FIBROBLASTS TRANSFORMED BY THE MUTANT P53<sup>VAL135</sup>

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The wild-type tumor suppressor gene p53 is known as a transcription factor in activating or suppressing target genes encoding proteins that regulate genome stability, DNA damage, cell arrest and apoptosis. However, the role of mutant p53 in the process of cell transformation is still unclear. Our recent work indicated that overexpression of mutant p53<sup>val135</sup> induced a high incidence of spontaneous transformation in prolonged cultures of Rat 6 fibroblasts. In order to identify genes related to neoplastic transformation induced by the mutant p53, the p53<sup>val135</sup>-overexpressor R6#13-8 and its derived spontaneously transformed cell line T2 were analyzed by mRNA differential display. In a systematic screening with 80 primer sets of reverse transcription polymerase chain reactions, three genes were found to be differentially expressed between R6#13-8 and T2 cells. Two genes, identified as homologues of the growth factor inducible immediate-early gene Cyr61 and the human nonmuscle myosin heavy chain-B

(nmMHC-B), were down-regulated in T2 cells. Interestingly, both genes were also suppressed in Rat 6 cells transformed by c-H-ras and v-myc, but not by v-src genes (JWP Yam *et al. Biochem Biophys Res Commun* 1999;266:472–80). Intriguingly, expression of the nmMHC-B gene effectively reversed the transforming phenotypes and the tumorigenicity of T2 cells, suggesting that the nmMHC-B may act as a tumor suppressor gene (JWP Yam *et al., Oncogene*, in press). Work is in progress to determine the tumor suppression potential of the Cyr61 gene in the transformed T2 cells.

### P3 NOV PROMOTES PROLIFERATION AND EARLY MYOGENIC DIFFERENTIATION OF 10T1/2 FIBROBLASTS

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To investigate the effects of NOV on cell growth, proliferation and differentiation, a full length version of the mouse gene (FLNOV) and a shorter version ( $\Delta$ NOV), which lacks the N-terminal secretory signal and the IGF binding protein (IGFBP) domain, were expressed in 10T1/2 fibroblasts under the control of a strong cytomegalovirus (CMV) promoter. Our results show that over-expression of FLNOV in stable selected cells leads to a dramatic increase in the rate of cell proliferation and cell growth. In contrast, these processes are inhibited in cell lines over-expressing  $\Delta$ NOV. We also find that both FLNOV and  $\Delta$ NOV promote the differentiation of 10T1/2 fibroblasts down the myogenic pathway, even in the absence of treatment with the inducer 5 aza-cytidine. Stable transfected bulk cultures expressing FLNOV express the myogenic genes myogenin and MyoD, but continue to proliferate and consequently fail to undergo terminal differentiation, even on serum starvation. In contrast,  $\Delta$ NOV stable transfectants behave similarly to 10T1/2 cells expressing a myogenin transgene, in that terminal muscle differentiation is observed, characterized by cell cycle exit, fusion of myoblasts to form multi-nucleate myotubes and expression of the terminal marker muscle creatine kinase.

### P4 IMMUNOCYTOCHEMICAL LOCALIZATION OF NOVH PROTEIN AND ULTRASTRUCTURAL CHARACTERISTICS OF NCI-H295R CELLS

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Biochemical and immunocytochemical results on NOVH protein secretion and localization in NCI-H295R cells are presented, as well as results on the ultrastructural characteristics of NCI-H295R cells. Ultrastructurally, the NCI-H295R cells are characterized by small quantities of rough and smooth endoplasmic reticulum, many free ribosomes, large nuclei with prominent nucleoli, numerous elongated mitochondria, a few Golgi complexes, and a small number of lipid droplets. Large numbers of coated pits and coated vesicles are found, but no secretory granules or exocytotic profiles are seen. Best ultrastructural preservation of NCI-H295R cells was achieved when fixation was done directly on the culture dishes and the cells were detached by scraping. Addition of affinity purified K19M antibody to culture dishes before processing NCI-H295R cells for ultrastructural examination results in high numbers of cells grouped together. Our biochemical results show that NCI-H295R cells secrete large amounts of NOVH protein. The immunocytochemical localization of NOVH protein, using the affinity purified K19M antibody, shows that the protein was localized in the cytoplasm, the plasma membrane and the nuclear envelope. This localization pattern, along with the ultrastructural and biochemical findings, raise interesting questions on the function(s) and the mode of secretion of NOVH protein.

**P5 NOVEL MODELS OF CTGF TRANSGENESIS IN VIVO: RECOMBINANT ADENO-ASSOCIATED VIRAL (RAAV) MEDIATED DELIVERY OF THE CTGF GENE**

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The CCN family of genes participate in a wide variety of biological processes including adhesion, migration, mitogenesis, differentiation and survival. More specifically, connective tissue growth factor (CTGF) stimulates connective tissue cell proliferation, extracellular matrix (ECM) synthesis, and fibroblast proliferation and is involved in fetal development. CTGF has also been implicated in skin fibrosis, atherosclerosis, wound healing, and many fibrotic disorders. Additionally, CTGF has been suggested as a possible downstream target for transforming growth factor  $\beta$  (TGF- $\beta$ ) in the induction of fibrosis. However, despite much circumstantial data, there is no direct link between CTGF expression and the onset or progression of fibrosis in a specific organ. To study the connection between CTGF and its biological properties in vivo such as fibrosis, we have developed a recombinant adeno-associated viral (AAV)-based approach. AAV is quickly becoming a popular vector for gene therapy based on many favorable properties including its (a) low immunogenicity; (b) lack of pathogenicity; (c) high stability of transgene expression; (d) ability to transduce terminally differentiated and non-dividing cells; (e) ease of purification and concentration; and (f) ability to deliver foreign DNA to a wide range of mammalian cells.

cDNA encoding full length (38 kDa) CTGF under the cytomegalovirus (CMV) promoter was cloned into a triple-play vector containing flanking AAV inverted terminal repeats, rep and cap genes, and neo<sup>r</sup>. This vector was then transiently transfected into HeLa cells and tested for both CTGF protein production by immunoprecipitation as well as its ability to produce infectious virus by a passage assay into C12 cells, as measured by Southern blot for viral DNA and immunoprecipitation of CTGF protein. Having verified the feasibility of this approach, the same strategy was adopted under G418 selection to isolate stable producer cell lines. Each stable HeLa cell line was examined for its ability to produce rAAV-CTGF virus, as described above, and positive cell lines were evaluated for viral titer by real time polymerase chain reaction. The cell line exhibiting the highest titer was expanded into a cell cube to facilitate efficient large-scale production of rAAV-CTGF. Once purified, rAAV-CTGF will be delivered to key target organs such as liver, kidney, eye, uterus, lung, and/or cardiovascular system to establish its physiological and pathological functions in vivo.

**P6 ESTABLISHMENT OF A RECOMBINANT CTGF EXPRESSION SYSTEM IN VITRO THAT MODELS CTGF PROCESSING IN VIVO: STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF MULTIPLE MASS CTGF PROTEINS**

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The primary translational product of human connective tissue growth factor (CTGF) is predicted to comprise 349 residues which, after cleavage of the signal peptide, would be expected to produce a protein of 323 residues with a  $M_r$  of 38 000. Recently, we have shown that following its synthesis in the pig reproductive tract, 38 kDa CTGF undergoes rapid but limited proteolysis yielding very stable 16–20 kDa isoforms that commence at Asp<sup>186</sup>, Ala<sup>197</sup>, and Cys<sup>199</sup> as well as 10 kDa isoforms that commence at Glu<sup>247</sup> or Glu<sup>248</sup>. The N-termini of these proteins commence at sites that are between modules II and III (16–20 kDa CTGFs) or between modules III and IV (10 kDa CTGF) suggesting that the inter-modular regions are protease-sensitive. These data suggest that low mass CTGFs, as well as the intact full length protein, may be biologically active in some circumstances.

In view of the difficulty of obtaining and purifying large quantities of multiple mass CTGF proteins from native sources, we attempted to produce them using recombinant DNA technology. While various strategies were attempted, the most successful was one in which cDNA encoding full-length 38 kDa human CTGF was cloned into the mammalian expression vector pcDNA3.1 and transfected into Chinese hamster ovary (CHO) 745 cells that are mutant for heparan sulfate and chondroitin sulfate synthesis. A limiting dilution was used

to isolate stably transfected clones which were then screened for recombinant CTGF expression by western blotting of conditioned medium. Several clones were shown to produce not only 38 kDa CTGF but also low mass (10–20 kDa) CTGFs as well. The clone with the highest level of expression was selected for large scale production of the recombinant proteins from serum-free medium. Conditioned medium was collected from transfected cells and subjected to heparin-affinity chromatography fast protein liquid chromatography (FPLC). All mass forms of CTGF (38 kDa, 16–20 kDa, 10–12 kDa) were shown to be heparin-binding and were subsequently separated from one another using a combination of gel filtration FPLC, cation-exchange FPLC and/or reverse-phase high performance liquid chromatography (HPLC). Structural analysis of the purified proteins showed that they commenced at Ala<sup>181</sup> (20 kDa), Leu<sup>184</sup> (18 kDa), Ala<sup>197</sup> (16 kDa) and Gly<sup>253</sup> (10 kDa). A single preparative run, representing purification from approx 4 L of conditioned medium, produced 1–2 mg of 16–20 kDa CTGFs, 100  $\mu$ g 38 kDa CTGF and 50–75  $\mu$ g 10 kDa CTGF. Additionally, we showed that pig uterine luminal fluids were able to digest 38 kDa CTGF to stable 16–20 kDa proteins, a phenomenon that was blocked by anti-thrombin III. Thrombin treatment of 38 kDa CTGF produced a 16 kDa CTGF isoform that commenced at Asp<sup>198</sup>. These data implicate serine proteases as one class of enzyme that can cause limited proteolysis of CTGF. While mitogenic activity associated with each isoform was variable and requires further analysis, all of the different mass forms of CTGF stimulated dose-dependent adhesion of Balb/c 3T3 cells between 5 and 20  $\mu$ g/ml. These results show that cell adhesion motifs in CTGF are located in the C-terminal region of the CTGF molecule. In conclusion, we have developed an in vitro expression system that closely mimics physiological CTGF processing and is highly amenable for future studies of the individual biological and cell binding properties of each CTGF isoform.

**P7 NOV IS EXPRESSED DURING IN VITRO DIFFERENTIATION OF CHICKEN MUSCULAR SATELLITE CELLS**

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Previous observations have established that the expression of nov is associated with heterotypic muscular differentiation in Wilms tumors and with striated muscle differentiation in normal developing chicken embryo.

In order to study further the role of nov in the normal muscular differentiation process, we have made use of muscular satellite cells.

Muscular satellite cells are small pluripotent cells lying between the basal membrane and the cytoplasmic membrane of myocytes. They are responsible for postnatal growth and regeneration of skeletal muscle. Their progeny can fuse to form new muscle cells following injury. Under normal conditions, the satellite cells are held in a quiescent state and constitute a pool of stem cells for the production of terminally differentiated muscular cells.

Differentiation of satellite cells can proceed ex vivo. In primary cultures, satellite cells proliferate during 12 to 18 days until they reach confluence. The first binucleated cells are observed from day 2 onwards and large multinucleated cells (myotubes) appear from day 4 to day 18.

After isolation, by enzymatic extraction, muscular satellite cells for muscle pectoralis major of three-day-old chicken were plated at a density of 14 200 cells/cm<sup>2</sup>. For clonal culture, the cell suspension was diluted and cells were plated at a concentration of 1 cell/well.

The expression of nov was followed by immunofluorescence and immunocytochemistry. Polyclonal nov antibodies were raised against a glutathione s-transferase (GST) fusion protein containing a C-terminal peptide specific for the chicken NOV protein. The nov antibodies were purified by two step affinity chromatography on protein-G and GST columns.

Our results indicated that, after two days in culture, some cells exhibited cytoplasmic labelling, usually with a multifocal pattern. As differentiation proceeded, the expression of nov was found to increase, the labelling being noticed in the cytoplasm all around the central nucleus or around the nuclei in multinucleated cells.

Co-labelling with anti-desmin indicated that NOV and desmin were expressed simultaneously. No overlap was observed between nov (cytoplasmic around the nuclei) and desmin (more peripheral) expression patterns.

Because satellite cells undergo all steps of normal differentiation in a short period of time, we believe that these cultures provide a good system in which to study in greater detail the relationship between nov expression and muscular differentiation.

#### **P8 TUMORIGENICITY OF HIGH GRADE RENAL CELL CARCINOMAS AND NOV EXPRESSION**

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The NOV gene is overexpressed in some tumors, such as in Wilms tumors with stromal components. The renal cell carcinomas (RCCs) of papillary subtype present several similarities with Wilms tumors: their origin from nephrogenic rests, their morphology, the duplication of the long arm of chromosome 7 as the first cytogenetic event and their multifocal feature. We investigated the expression of the NOV gene in the papillary subtype of RCCs.

A fine analysis of karyotype was performed on 13 xenografted RCCs. Five tumors have the cytogenetic characteristics of the papillary subtype with duplication of the 7q31 and 8q22-pter minimal regions. These tumors have a tumor growth rate in mice that is twice shorter than conventional tumors which present a loss of 3p and of the 9p21 and 14q regions known to be markers of worse prognosis. In fast growing tumors, we found a level of NOV mRNA and of secreted protein significantly higher than the other tumors.

Our results suggested that the expression of a secreted cell proliferation regulatory protein such as NOV might be important in tumor development. The level of this released protein in our fast growing tumors could be high enough to induce a significant autocrine effect or more probably a paracrine effect on surrounding cells, and might thus modify the interactions between tumor cells and the extracellular matrix.

#### **P9 POTENTIAL FUNCTIONAL INTERACTIONS OF THE NOVH C-TERMINAL DOMAIN IN NORMAL AND TUMOR TISSUES FROM ADRENAL CORTEX**

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Experiments performed in our laboratory have established that novH is a marker of tumor differentiation in several tumor types.

In order to gain insight into the biological activity of novH and the functionality of the molecular structural domains, we have recently used a two hybrid system to identify proteins interacting with novH. We established that: (1) the full length novH binds fibulin 1C, a protein of the extracellular matrix interacting with several growth factors (B Perbal *et al. Proc Natl Acad Sci* 1999;96:869-74); (2) naturally occurring truncated form of novH exhibits a high fibulin 1C affinity. These observations represented the first clues for novH being involved in cell adhesion regulation and led us to hypothesize that the production of truncated isoforms of novH might be a critical aspect in the modulation of its biological activity.

The multimodular structure of the NOV and other CNN proteins raises interesting questions as to the participation of each individual module conferring the biological properties to the full length proteins. Either the biochemical functions of the insulin-like growth factor binding protein (IGFBP), Von Willebrand type (VWC), thrombospondin (TSP) and carboxy-terminal module (CT) modules contained in these proteins are indeed conserved and sum up in the full length protein, or the presence of each module confers on the whole protein specific biological function(s) which may substitute or add upon those of individual modules. The CT of the CNN proteins which is supposed to represent a dimerization domain contains a cysteine-knot motif and is involved in the dimerization of several growth factors such as nerve growth factor (NGF), transforming growth factor  $\beta$ 2 (TGF- $\beta$ 2) and platelet derived growth factor BB (PDGFBB).

In the present study we have used an immunological approach to establish the cellular distribution of NOV in normal adrenal, adenomas, and adrenocortical tumors. We have established that the human adenocortecoid NCI H259R cells (American Type Cell Collection) expressed and secreted high levels of the 48 kDa NOVH protein and a significant amount of a truncated 30 kDa NOV isoform. By performing enzyme linked immunosorbent assay, we could establish that the CT of NOV which appears to be involved in protein cross talk is not freely accessible in the native NOV protein, therefore suggesting that it is masked as a result of protein folding or involved in high affinity interactions with other partners when it is secreted.

#### **P10 LOCALIZATION OF CONNECTIVE TISSUE GROWTH FACTOR IN NORMAL REGENERATING AND DENERVATED NON-REGENERATING SCARRING LIMB STUMPS OF ADULT NEWTS**

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Adult urodele limb regeneration provides a unique model for studying tissue dedifferentiation and redifferentiation in a scarless environment. However, following denervation, the normal restorative process is aborted and a scar is formed as part of the healing process. Connective tissue growth factor (CTGF) is a recently described cytokine that is implicated in normal physiological processes such as embryogenesis, angiogenesis, and extracellular matrix remodeling. CTGF over-expression, however, is associated with fibrotic disorders in major organs of the mammals including humans. Bilateral forelimb amputations were performed on newts at either the mid stylo- or zeugopodium levels. Regenerating limb amputees were harvested at representative time points covering the entire regeneration cascade. Denervation, by severing the brachial plexus, of regenerating limbs was done at either nerve-dependent (early regeneration bud) or nerve-independent (palette and early digitiform) stages. Specimens were fixed and processed for either CTGF immunostaining with anti-human CTGF peptide antibody (78% homologous with the newt sequence) or digoxigenin labeled CTGF riboprobe. By the early healing phase of regeneration CTGF was localized in white blood cells, Schwann's cells, and stump skeletal muscles. At the pre-blastemic and blastemic phases, positive CTGF expression became evident in the basal layer of the wound epithelium, blastema cells, osteoclasts, and skeletal muscle fibers. During redifferentiation and the morphogenetic phase, CTGF was confined to the wound epithelium at regions of indentation between future digits and in redifferentiated hypertrophied cartilage. Following denervation CTGF become over-expressed in the nerve stump, periosteum, and atrophied skeletal muscle fibers. Meanwhile, CTGF expression was suppressed in osteoclasts in the nerve-dependent phase of regeneration. These data support a role for CTGF in the normal processes of inflammatory responses, extracellular matrix remodeling, cellular proliferation, endochondral ossification, and natural cell death morphogenesis during urodele limb regeneration cascade. Over-expression of CTGF following denervation is correlated with the loss of normal tissue regeneration and the appearance of scarring environment.

#### **P11 EXPRESSION OF CTGF AND TGF- $\beta$ 1 IN THE PSEUDOPREGNANT MOUSE UTERUS**

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Connective tissue growth factor (CTGF) is multifunctional in normal physiological processes as well as pathological disorders. Previously we have demonstrated that in the mouse uterus, CTGF is expressed principally in epithelial cells but that the time of implantation is associated with decreased epithelial staining (prior to epithelial erosion) and increased expression by differentiating fibroblast cells in the decidual zone. The present study used pseudopregnant animals to answer the question of which aspects of CTGF expression are not dependent on the presence of an active blastocyst. Pseudopregnancy was established using standard methods whereby fertile females were mated with vasectomized males (vaginal plug = day 0.5). The distribution of CTGF and transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) was analyzed on days 0.5-5.5 using *in situ* hybridization and/or immunohistochemistry. In the luminal and glandular epithelial cells, staining for CTGF protein and mRNA was strong on days 0.5-2.5, somewhat

weaker on days 3.5–4.5 and higher again on day 5.5 (recycling day). The expression pattern of CTGF in epithelial cells was paralleled by TGF- $\beta$ 1 protein. In the stroma, CTGF mRNA was not detected on days 0.5–3.5 while staining for CTGF protein ranged from weak to moderate suggesting that it originated from an alternative source such as epithelium. On day 4.5, stromal CTGF mRNA levels were elevated and associated with extracellular matrix remodeling and extensive neovascularization. Stromal CTGF levels were lower on day 5.5. Over this time course, levels of stromal TGF- $\beta$ 1 were moderate to high but not always correlated with CTGF levels. Myometrium exhibited no or very low levels of CTGF and TGF- $\beta$ 1. These data show that CTGF and TGF- $\beta$ 1 are expressed in the pseudopregnant mouse uterus in a manner comparable to that described for normal pregnancy and the estrous cycle. At present, we cannot rule out a role for blastocyst-derived factors in the changes in CTGF production that are associated with the processes of implantation and decidualization. Nonetheless, the data show that prior to arrival of an active blastocyst at its implantation site, maternal factors are the primary cues for CTGF expression in the uterus. Such factors are likely to include ovarian steroids. Accordingly, the inter-relationship between CTGF, TGF- $\beta$ 1 and steroid hormones is currently under investigation, as is the relationship of the implanting blastocyst to local changes in uterine CTGF production.

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## Papers

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### A1 THE CCN PROTEINS: MULTIPOTENT MATCHMAKERS?

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The prototypic members of the CCN family (CTGF, CYR61 and NOV) were discovered in the early 1990s. Additional members of the family have been identified, including Elm-1/WISP-1, WISP-3 and Cop-1/WISP-2. These highly conserved cysteine-rich proteins share four modular domains (each encoded by a single exon) with sequence similarities to insulin-like growth factor binding protein (IGFBP), von Willebrand factor, thrombospondin (TSP), and a cysteine knot characteristic of some growth factors including platelet derived growth factor (PDGF), nerve growth factor (NGF) and transforming growth factor  $\beta$  (TGF- $\beta$ ). Cop-1/WISP-2 is unique as it lacks the carboxy-terminal cysteine knot domain.

Purified CCN proteins have been demonstrated to mediate and promote cell adhesion, migration, proliferation, and survival. As matrix-associated, heparin-binding proteins, CTGF and CYR61 are novel ligands of integrins  $\alpha$ v $\beta$ 3 and  $\alpha$ IIb $\beta$ 3, and NOV interacts with fibulin 1C, suggesting their involvement in cell adhesion signaling. Both CTGF and CYR61 induce angiogenesis *in vivo* and chondrogenesis *in vitro*. CTGF is expressed in fibroblasts during wound healing and can induce fibrosis *in vivo*. Furthermore, CTGF has been demonstrated to mediate both the mitogenic and matrigenic activities of TGF- $\beta$ . Other studies have revealed that CYR61 promotes tumor growth, whereas Cop-1 or Elm-1/WISP-1 can inhibit tumor growth.

The multimodular structure of the NOV and other CCN proteins raises interesting questions as to the participation of each individual module on conferring the biological properties to the full length proteins. Either the biochemical functions of the IGFBP, Von Willebrand type C (VWC), TSP and carboxy-terminal modules contained in these proteins are indeed conserved and sum up in the full length protein, or the presence of each module confers on the whole protein specific biological function(s) which may substitute or add upon those of individual modules.

Studies performed with CTGF and NOV have highlighted the importance of post-translational processing of the CCN proteins and the involvement of CCN isoforms in different biological pathways.

The complex pattern of CCN gene expression in cancer cells also suggested that the biological activities of CCN proteins might be dependent upon their interactions with other proteins and ligands whose identity is presently being uncovered.

Expression and possible roles of CCN proteins in the control of cell proliferation in normal and cancer cells will be discussed with respect to their conserved multimodular organisation and their cellular sublocalisation.

### A2 THE EXPRESSION OF CONNECTIVE TISSUE GROWTH FACTOR DURING WOUND HEALING

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Connective tissue growth factor (CTGF) is a 38 kDa cysteine-rich protein which is exclusively induced by transforming growth factor  $\beta$  (TGF- $\beta$ ) in fibroblasts, having chemotactic and mitogenic activity for fibroblasts. We reported gene expression of CTGF in fibrotic diseases (ex. PSS and keloid) and tissue regeneration in a subcutaneous wound model, but there have been no reports that mention CTGF expression in real open wound sites (ulcers). In this study, we investigated CTGF expression in wound healing. We made ulcers on the forearm of human volunteers, using 4 mm Dispo-punch. Tissue materials were taken on days 1, 3, 7, 10, and 16 until the re-epithelization was completed. These tissues were used for *in situ* hybridization using digoxigenin labeled CTGF probes. We also tested rat skin wounds. The results revealed that no CTGF message was detected in normal skin. On day 3, small amounts of message were found in dermal fibroblasts. On days 7 and 10, large amounts of CTGF message were detected in granulation tissue fibroblasts. On day 16, the CTGF message was decreased. Similar results were obtained from the rat wounds. Our results indicate a correlation between CTGF and granulation formation. Also we found the CTGF message on the endothelial cells of the vessel at the wound site.

### A3 IN VITRO EVIDENCE FOR DIFFERENTIAL INVOLVEMENT OF CTGF, TGF- $\beta$ AND PDGF-BB IN MESANGIAL RESPONSE TO INJURY

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**Background:** Connective tissue growth factor (CTGF) is a profibrotic growth factor, which is up regulated in wound healing and renal fibrosis, including anti-Thy-1.1 nephritis. The kinetics of CTGF mRNA expression in anti-Thy-1.1 nephritis suggested that CTGF regulation might contribute to the glomerular response to injury, downstream of transforming growth factor  $\beta$  (TGF- $\beta$ ). In anti-Thy-1.1 nephritis, the initial damage is followed by mesangial repair and limited sclerosis, which involves mesangial cell activation ( $\alpha$  smooth muscle actin ( $\alpha$ SMA) expression), proliferation, migration and extracellular matrix (ECM) production. The present *in vitro* study addresses the possible role of CTGF in these different aspects of mesangial response to injury, and how CTGF activity might relate to effects of TGF- $\beta$  and platelet derived growth factor BB (PDGF-BB).

**Methods/Results:** Immunostaining and enzyme linked immunosorbent assay (ELISA) showed that  $\alpha$ SMA expression and transformation of mesangial cells into myofibroblast-like cells was induced by TGF- $\beta$ , but not affected by PDGF-BB, CTGF or neutralizing anti-CTGF antibodies. <sup>3</sup>H-thymidine incorporation and Ki67 staining demonstrated that, unlike PDGF-BB, neither CTGF, nor TGF- $\beta$  induced the proliferation of mesangial cells. In contrast, both CTGF and TGF $\beta$  induced mesangial cell migration, as evidenced by approximation of wound edges in scrape-wounded, non-proliferating rat mesangial cell monolayers. Also fibronectin (FN) expression was upregulated by both CTGF and TGF- $\beta$ , as measured by dot-blot analysis. Anti-CTGF completely blocked the effect of added CTGF. Moreover, anti-CTGF significantly reduced TGF- $\beta$ -induced increase in FN.

**Conclusions:** It thus appears that CTGF is specifically involved in a subset of the adaptive changes of mesangial cells involved in mesangial repair and sclerosis, which makes it an interesting candidate target for future intervention strategies.

**A4 MECHANICAL FORCE-INDUCED CYR61 GENE EXPRESSION IN SMOOTH MUSCLE CELLS**

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Mechanical forces are key environmental cues that control smooth muscle cell growth, differentiation and survival. Conceptually, mechanical forces are sensed by several putative mechano-receptors in the cellular membrane including ion channels, integrins, G-protein and growth factor receptors and transduced into the cell upon the release of second messenger molecules and activation of specific signaling pathways.

Consequently, specific sets of muscle and non-muscle specific genes are activated which affect the phenotypic features of the cells. However, the full repertoire of strain-inducible genes is not completely defined and the biological significance of specific gene alterations is a yet unresolved issue. The present study was designed to explore a potential regulation of the prototypic member genes of the CCN family, cystein-rich protein 61 (cyr61) and connective tissue growth factor (CTGF), by mechanical forces in smooth muscle cells. Both cyr61 and CTGF are factor-inducible immediate early genes encoding secreted extracellular signaling molecules with presumed functions in cell migration, adhesion, proliferation and survival. In our experiments, we used an in vitro mechanical device that allows the application of a cyclic biaxial strain to the cells cultured on a deformable substrate and assessed the gene expression levels of cyr61 and CTGF. We found that mechanical stimulation of the cells induced rapidly a burst of cyr61 transcripts (6–10-fold) and a simultaneous alteration of those of CTGF. While the changes in cyr61 expression were transient (up to one hour) and proportional to the magnitude of the applied strain, those of CTGF were sustained throughout the time period of mechanical stimulation. Internal transduction mechanisms involving protein kinase C and phosphatidyl 3-kinase activation partly suppress cyr61 gene responsiveness to mechanical stretch. Our results establish that the expression of cyr61 is under the control of mechanical stimuli in smooth muscle cells and predict a potential key role of this protein in the mechanotransduction and/or the phenotypic modulation processes in the cells.

**A5 INDUCTION OF CTGF IN RENAL CELLS: REGULATORY ROLE OF THE SMALL G-PROTEIN RHOA AND THE ACTIN CYTOSKELETON**

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Expression of connective tissue growth factor (CTGF) was investigated in renal cells, primary cultures of rat mesangial cells and a human renal fibroblast cell line. Induction of CTGF mRNA was observed after treatment of the cells with transforming growth factor  $\beta$  (TGF- $\beta$ ), but also by activation of heptahelical receptors by lysophosphatidic acid (LPA). Induction of CTGF mRNA by LPA was transient with maximal expression after one to two hours, whereas induction of CTGF by TGF- $\beta$  increased over time.

Inhibition of proteins of the Rho family by toxin B abrogated basal as well as CTGF expression stimulated by LPA and TGF- $\beta$ . Inhibition of the downstream mediator of RhoA, the ROCK kinase, by Y27632 partially reduced induction of CTGF. Toxin B not only affected gene expression, but disrupted the actin cytoskeleton, as observed after treatment with cytochalasin D. Disassembly of actin stress fibers by cytochalasin D partially reduced basal and stimulated CTGF expression. These data indicate that an intact actin cytoskeleton is crucial for the expression of CTGF. Elimination of the input of Rho proteins by toxin B, however, was significantly more effective and their effect on CTGF expression thus goes beyond disruption of the cytoskeleton.

These findings thus establish activation of heptahelical receptors coupled to pertussis toxin-insensitive G proteins as a novel signaling pathway to induce CTGF in renal cells. Proteins of the Rho family and an intact cytoskeleton were identified as critical determinants of CTGF expression induced by all stimuli tested.

**A6 CONNECTIVE TISSUE GROWTH FACTOR REGULATION AND EXPRESSION IN HUMAN GINGIVAL CELLS AND TISSUES SUGGESTS A ROLE IN PHENYTOIN-INDUCED GINGIVAL OVERGROWTH**

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Gingival overgrowth occurs as a side effect of certain drug therapies, and is often treated by periodontal surgery to allow for improved oral hygiene and prevention of serious microbial oral and systemic infection. The most notable causative drugs are phenytoin, nifedipine, and cyclosporin A. Transforming growth factor  $\beta$  (TGF- $\beta$ ) is known to be elevated in certain gingival overgrowth tissues, and the present study first assessed TGF- $\beta$  regulation of extracellular matrix production by gingival connective tissue cells. Results show that 4–400 pM TGF- $\beta$  increased collagen and lysyl oxidase mRNA levels and lysyl oxidase enzyme activity two to fourfold in 24–48 hours. This is in contrast to the more rapid regulation observed in murine osteoblastic cell cultures. The regulation of connective tissue growth factor (CTGF) by TGF- $\beta$  in gingival fibroblast cultures was then investigated. CTGF mRNA levels were rapidly increased up to 20-fold by 4–400 pM TGF- $\beta$ , and increased levels of CTGF protein were secreted by human gingival fibroblasts. Exogenously added recombinant human CTGF increased insoluble collagen accumulation and lysyl oxidase enzyme activity levels, but no detectable increases in collagen or lysyl oxidase mRNA levels were found. Anti-CTGF antibodies did not block TGF- $\beta$  stimulated collagen mRNA levels. These studies show that CTGF appears to contribute to extracellular matrix accumulation by pathways that complement rather than fully mediate the effects of TGF- $\beta$  in human gingival fibroblast cultures. New quantitative computer assisted immunohistochemistry studies of human gingival overgrowth tissues for CTGF demonstrate a remarkably high level of CTGF detected in vivo in deep gingival connective tissues in phenytoin induced gingival overgrowth (n = 9). Little CTGF staining was found in cyclosporin A (n = 5) and nifedipine-induced gingival overgrowth tissues (n = 4), and non-hyperplastic control tissues (n = 9) were negative for CTGF. These assays which show for the first time that CTGF is increased in fibrotic phenytoin-induced gingival overgrowth tissues suggest unique cytokine profiles in phenytoin-induced gingival overgrowth, and imply a role for CTGF in the development of fibrotic gingival lesions.

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**A7 EXPRESSION OF THE HUMAN NOV GENE IN FIRST TRIMESTER FETAL TISSUES**

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Using in situ hybridisation and immunocytochemistry we have examined the expression of the human Nov gene in sections of human embryos from four to 8 weeks of gestation. The study reveals widespread expression in derivatives of all three germ layers. The most abundant sites of expression are in the spinal cord, adrenal cortex, fusing skeletal and smooth muscle, the urogenital system and the developing heart. Additionally, expression is seen in the cranial ganglia, differentiating chondrocytes, gonads, and lung. The sites of expression suggest strongly that autocrine or paracrine expression of Nov is associated with the process of cell differentiation. At the early stages studied here, the pattern of expression of NOV mRNA in the developing spinal cord is very specific and is limited to the floor plate and the ventral horns. The expression in the ventral horns is clearly neuronal and from anatomical criteria appears to be in motor neurons. This descriptive study indicates organ systems and processes which may be particularly dependent on NOV expression for their

controlled proliferation and differentiation during key phases of organogenesis. An emerging common site of expression for the CCN family members is the developing musculo-skeletal system and the role of Nov in the differentiation of muscle and cartilage will be discussed.

#### A8 EXPRESSION AND REGULATION OF HCYR61 IN HUMAN BONE CELLS

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The human cysteine-rich protein 61 (hCYR61) is a member of an emerging gene family important for cellular processes such as angiogenesis, wound healing, cell proliferation and differentiation. Previously, we identified hCYR61 as a fast and transiently regulated gene by differential display polymerase chain reaction (PCR) in a human osteoblast cell-line (hFOB-cells).

RNA was isolated from hFOB cells (kindly provided by Dr Spelsberg, Mayo Clinic, USA) and analysed for hCYR61 mRNA expression by northern blotting. Protein levels were analysed from cellular extracts by western blotting. Intracellular localisation of hCYR61 was studied following transient expression of a green fluorescent protein (GFP)-hCYR61 fusion protein in hFOB-cells and primary osteoblasts. Immunohistology and in-situ hybridisation were performed using hFOB-cells and primary osteoblasts of different maturation stages and using bone samples.

hCYR61 mRNA was found to be markedly upregulated by factors important for bone metabolism such as 1,25(OH)<sub>2</sub>-vitamin D<sub>3</sub> (10 nM) and the growth factors tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) (10 nM), epidermal growth factor (EGF) (20 ng/ml), basic fibroblast growth factor (bFGF) (3 ng/ml) and interleukin 1 $\beta$  (IL1 $\beta$ ) (3 ng/ml) within one hour of treatment. At the protein levels the same factors upregulated hCYR61 2–3 threefold (1,25(OH)<sub>2</sub>-vitamin D<sub>3</sub> 10 nM) or 5–10 fold (TNF $\alpha$  10 nM, EGF 20 ng/ml, bFGF 3ng/ml and IL1 $\beta$  3 ng/ml) within 24 to 48 hours as studied from cellular extracts of hFOB-cells. Supernatants of untreated and growth factor treated hFOB cells again showed a 5–10 fold upregulation of secreted hCYR61 protein. A full length hCYR61 protein C-terminally fused to GFP localized to the Golgi apparatus in hFOB-cells, primary osteoblasts and COS-7 cells. The N-terminal 34 amino acids of hCYR61 fused to GFP were sufficient to target the protein to the Golgi apparatus. Conventional in situ hybridisation revealed no detectable hCYR61 mRNA expression in normal bone; however, samples from fracture callus displayed significant hCYR61 expression in mesenchymal cells and osteoblasts. Similarly, using immunohistology samples from normal bone displayed little if any signal, whereas samples from fracture callus were highly positive in mesenchymal cells as well as on bone surfaces. Samples from a human growth plate indicated high hCYR61 expression in mature chondrocytes. Using hFOB-cells and primary osteoblasts of different maturation stages a high expression of hCYR61 protein in proliferating cells was observed using fluorescence coupled immunocytochemistry whereas differentiated cells revealed much lower signal intensity.

In summary, hCYR61 is expressed and regulated with characteristics of an early-response gene in human osteoblasts in vitro. The protein is directed towards the Golgi apparatus and secreted in a regulated manner. Also in human bone hCYR61 is expressed in situations of enhanced bone turnover/bone formation. Most likely, hCYR61 in human bone acts as an extracellular matrix signaling molecule and couples activities of different cell types.

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#### A9 NOV EXPRESSION AND REGULATION IN THE GROWTH PLATE AND IN CARTILAGE TUMOURS

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Chondrocytes in the growth plate proliferate, hypertrophy, and undergo terminal differentiation, associated with apoptotic cell death and the deposition of type X collagen as the scaffolding for new bone formation. Parathyroid hormone related protein (PTHrP) regulates the differentiation of the growth plate chondrocytes. Enchondromas are a benign cartilage lesion that can progress to chondrosarcoma, and behave as if they have their origin in growth plate chondrocytes that

have not undergone terminal differentiation. The human nephroblastoma overexpressed (novH) protein plays a role in cell differentiation during development (as demonstrated in the developing brain and kidney). It is therefore possible that novH also plays a role in the differentiation of growth plate chondrocytes. Human growth plate specimens (obtained from patients undergoing limb shortening operations) and cartilage tumours samples (obtained at the time of surgical resection) were processed to extract RNA and protein, and were established as organ cultures. NovH expression was determined in the primary tissue samples using western analysis and reverse transcription polymerase chain reaction. Organ cultures were used to determine the effect of treatment with PTHrP on the expression of novH.

We found that the growth plate samples all expressed nov. Lower grade (more differentiated) cartilage lesions also expressed nov, while the higher grade (less differentiated) exhibited variable expression. Treatment of the organ cultures with PTHrP resulted in an increase in nov expression. These data suggest that nov plays a role in the differentiation of chondrocytes.

#### A10 IDENTIFICATION AND CHARACTERIZATION OF CTGF-L AS A NOVEL MODULATOR OF OSTEOBLAST AND CHONDROCYTE FUNCTIONS

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Connective tissue growth factor-like (CTGF-L) protein is a novel member of the cysteine-rich CCN (Cyr61, CTGF, NOV) family that contains multiple domains. Both human and murine CTGF-L proteins contain the IGF binding domain, the Von Willebrand factor type C domain, the thrombospondin type I domain but lack the carboxy terminal domain found in other CCN members. CTGF-L mRNA (~1.3 kb) is expressed in primary human osteoblasts, fibroblasts, ovary, testes and heart and an approximately 26 kDa protein is secreted from primary human osteoblasts and fibroblasts. In situ hybridization indicates high expression in osteoblasts forming bone, discrete alkaline phosphatase positive bone marrow cells and chondrocytes. The expression of CTGF-L mRNA also increases as primary human osteoblasts mineralize in vitro. Recombinant CTGF-L binds to both insulin-like growth factors and  $\alpha$  $\beta$  integrin and exhibits activities in several in vitro assays. Adhesion of primary human osteoblasts and osteosarcoma cell lines (MG 63 and Ros 17/2.8) to plastic wells coated with recombinant CTGF-L is enhanced to an extent comparable to that reported with vitronectin. CTGF-L also stimulated mineralization of human osteoblasts in vitro. Furthermore, recombinant CTGF-L stimulates proteoglycan synthesis in bovine chondrocytes. These data suggest an important role of CTGF-L in regulating osteoblast and chondrocyte functions, possibly by modulating the interaction of cells with the extracellular matrix.

#### A11 INTEGRINS MEDIATE CYR61-INDUCED ACTIVITIES IN HUMAN SKIN FIBROBLASTS

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Cyr61, an angiogenic factor and a member of the CCN gene family, is an extracellular matrix-associated heparin-binding protein which mediates cell adhesion, stimulates cell migration, and enhances basic fibroblast growth factor-stimulated cell proliferation in human skin fibroblasts. Cyr61 induces angiogenesis and is expressed in dermal fibroblasts during cutaneous wound healing. We have previously shown that Cyr61 is a ligand of, and binds directly to, the integrins  $\alpha$  $\beta$ 3 and  $\alpha$ IIb $\beta$ 3 on endothelial cells and blood platelets, respectively. Recently, we have demonstrated that adhesion of primary human skin fibroblasts to Cyr61 is mediated through integrin  $\alpha$ 6 $\beta$ 1 and cell surface heparan sulfate proteoglycans. Fibroblast adhesion to immobilized Cyr61, in the absence of other stimuli, results in  $\beta$ 1 integrin-containing focal complex formation, cytoskeletal reorganization, cell spreading with the formation of lamellipodia and filopodia, and activation of intracellular signaling molecules including focal adhesion kinase, paxillin, Rac, and mitogen activated protein kinase. These results show that Cyr61 can act as an adhesive signaling molecule through integrin  $\alpha$ 6 $\beta$ 1. In contrast to

cell adhesion, Cyr61 induced chemotaxis and enhanced basic fibroblast growth factor (bFGF) mitogenesis are mediated through different integrin receptors. Consequently, monoclonal antibodies against integrin  $\alpha v \beta 5$  block Cyr61 stimulated chemotaxis, and a monoclonal antibody (LM609) against integrin  $\alpha v \beta 3$  abrogates the enhancement of bFGF-stimulated fibroblast mitogenesis by Cyr61. Thus, the adhesion, migration, and pro-mitogenic activities of Cyr61 in skin fibroblasts are mediated through distinct integrin receptors. The differential utilization of different receptors by Cyr61 is most likely cell- and context-dependent, dictated by the extracellular milieu to achieve disparate biological consequences.

**A12 INTEGRIN  $\alpha_M \beta_2$  ACTS AS AN ADHESION RECEPTOR ON PERIPHERAL BLOOD MONOCYTES AND THP-1 CELLS FOR CYR61 AND CONNECTIVE TISSUE GROWTH FACTOR**

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Cyr61 and connective tissue growth factor (CTGF) are growth factor-inducible immediate-early gene products found in normal blood vessel walls, advanced atherosclerotic lesions and healing cutaneous wounds. We showed that the adhesion of endothelial cells, platelets and fibroblasts to these proteins is mediated by integrin receptors. Because monocyte adhesion is important for inflammation, wound healing and atherosclerosis, we examined the adhesion of isolated peripheral blood monocytes (PBMC) and THP-1 cells to Cyr61 and CTGF. Both PBMC and THP-1 cells adhered in a dose-dependent manner to Cyr61- and CTGF-coated microtiter wells. Moreover, stimulation of THP-1 cells with 20  $\mu$ M ADP caused a six to 10 fold increase in cell adhesion to both proteins. Time course studies showed that THP-1 cell adhesion to Cyr61 was transient, peaking at 20–30 minutes and declining thereafter. In inhibition studies, while EDTA completely blocked THP-1 cell adhesion to Cyr61, GRGDSP and echistatin had little effect. These data suggest the involvement of an RGD-insensitive integrin receptor. Using monoclonal antibodies specific for integrin subunits, we found that the adhesion of THP-1 cells and PBMC to Cyr61 and CTGF was specifically blocked by YFC118.3 (anti- $\beta_2$ ), and by 44a and 2LPM19c (anti- $\alpha_M$ ). In contrast, mouse IgG and 6S6 (anti- $\beta_1$ ) had no effect. Thus, the adhesion of monocytes to Cyr61 and CTGF is mediated primarily by integrin  $\alpha_M \beta_2$ . Consistent with the cell adhesion data, a glutathione S-transferase (GST)-fusion protein containing the I-domain of the  $\alpha_M$  subunit bound specifically to immobilized Cyr61 and CTGF, and the binding was completely blocked by 2LPM19c (anti- $\alpha_M$ ) but not by YFC118.3 (anti- $\beta_2$ ). Collectively, these results identified integrin  $\alpha_M \beta_2$  as an adhesion receptor on monocytes for Cyr61 and CTGF. The interaction of monocytes with these proteins may have important implications in the pathophysiological function of monocytes.

**A13 IDENTIFICATION OF WISP-1 BINDING FACTORS**

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WISP-1 (Wnt-1 induced secreted protein 1) is a member of the CCN family of growth factors which includes connective tissue growth factor, *cyr61*, *nov*, WISP-2 and WISP-3. Cell specific and tissue specific differences in the expression and function of different CCN family members suggest they have non-redundant roles. It was of interest to characterize the interaction of WISP-1 with the cell surface and to identify binding partners, which may help in the elucidation of its biological function. The distribution of WISP-1 binding sites was restricted to cell types with a fibroblastic phenotype. Using a solid phase assay, we showed that human skin fibroblast conditioned media contained a WISP-1 binding factor. Competitive inhibition with different glycosaminoglycans and treatment with specific glycosaminoglycan lyases and proteases demonstrated that the binding of WISP-1 was mediated by a dermatan sulfate proteoglycan. Mass spectrometric analysis identified the purified binding proteins as decorin and biglycan. Decorin and biglycan were shown to interact directly with WISP-1 and to inhibit its binding to human skin fibroblast conditioned media. Similarly, the binding of WISP-1 to human skin fibroblasts was inhibited in the presence of dermatan sulfate, decorin, biglycan or by treatment of the cell surface with dermatan sulfate specific lyases. Together these results demonstrate that WISP-1 possesses a specific binding site for dermatan sulfate that is

responsible for its interaction with decorin and biglycan as well as the surface of human skin fibroblasts. We propose that this specific interaction plays a role in the regulation of WISP-1 function.

**A14 ISOLATION OF A NOVEL GENE WHICH HAS LITTLE SIMILARITY WITH CCN FAMILY MOLECULES AND INTERACTS WITH WNT PATHWAY**

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In a functional screening seeking for molecules that are responsible for neural patterning, we isolated a novel clone (called 280) which can posteriorise neural explant in xenopus animal cap. A homology search showed that the 280 protein contains cysteine knots which are conserved in some secreted molecules such as members of the CCN family and mucin. Six per cent of the amino acid alignment (13/206, including four cysteines) was homologous to CEF10. 280 also contained predicted signal sequence and was indeed detected in the conditioned medium as well as in the expressing cells.

From the results of RNA injection into xenopus embryos, 280 was suggested to interact with wnt signalling. An immunoprecipitation study showed that 280 can bind to the putative wnt receptor Frizzled. In the neural explant 280 was able to activate wnt-signalling pathway. However, in a another context, such as in mesoderm and in gastrula embryos, 280 antagonised the activity of wnt, presumably by interfering with the wnt-frizzled interaction. A deletion construct that lacks the C-terminus including the region homologous to the CCN family members was not functional in terms of capability of inducing specific neural markers. We are examining further the functional role of the cysteine knots and its relation with the Wnt pathway.

**A15 WISP3 EXPRESSION IN EUKARYOTIC CELLS**

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We are interested in WISP3 because it is associated with the heritable skeletal disorder progressive pseudorheumatoid dysplasia (PPD). PPD is an autosomal recessive form of spondylo-epiphyseal dysplasia tarda, and is characterized by childhood-onset swelling and stiffness of joints, joint contractures, gradual loss of articular cartilage, and precocious arthritis. We have identified putative loss-of-function mutations in WISP3 among affected patients with PPD (for example, J Hurvitz *et al. Nat Genet* 1999;23:94–8). To understand the role of WISP3 during skeletal growth and homeostasis we have been exploring ways of studying WISP3 activity in vitro and in vivo. We have begun by expressing WISP3 in eukaryotic cells and by targeting the WISP3 gene in the mouse. To date we have performed transient transfections of Cos-7 cells and Chinese hamster ovary (CHO) cells using WISP3 constructs that have been tagged with c-myc and FLAG epitopes in the hinge-region domain of the polypeptide. Our preliminary results suggest WISP3 is a cell membrane-associated protein that is capable of binding to heparin and of forming higher molecular weight structures. We are beginning to express WISP3 in insect cells using baculovirus and we are trying to produce polyclonal antibodies against WISP3 using a prokaryote-expressed fragment and an oligopeptide antigen. We have been able successfully to target the WISP3 gene in mouse embryonic stem cells and to generate chimeric mice. Progeny from the chimeric mice are currently being screened for germline transmission; knockout mice may soon be available.

**A16 STRUCTURE AND FUNCTIONAL ANALYSIS OF CTGF**

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Fibrotic disorders represent the largest segment of human disease for the populations of North America, Europe and Japan. These disorders are characterized by an overproduction of connective tissue (primarily collagen) which interferes with the normal function of the tissue or organ. It is widely accepted that these disorders are initiated in response to some type of injury, which leads to the chronic inflammation and subsequent overproduction of growth factors that encourage the formation of connective tissue. One common growth factor

which has been demonstrated to be overproduced in all fibrotic disorders examined to date is transforming growth factor  $\beta$  (TGF- $\beta$ ). Cell culture studies have demonstrated that TGF- $\beta$  can stimulate both cell proliferation (mitogenic) and increased collagen synthesis (matrigenic) responses in connective tissue cells. Most investigators feel that TGF- $\beta$  is one of the prime initiators of connective tissue formation in fibrotic disorders. During the course of our studies on the mechanism whereby TGF- $\beta$  stimulates connective tissue formation, we discovered another growth factor, connective tissue growth factor (CTGF). Using a variety of methods including anti-sense CTGF RNA, anti-CTGF antibodies and agents that block TGF- $\beta$  induction of the CTGF gene, we have determined that all the mitogenic and matrigenic activities of TGF- $\beta$  are mediated in part by CTGF dependent pathways. Thus, agents that block CTGF synthesis or action are effective inhibitors of TGF- $\beta$  induced fibroblastic cell proliferation and extracellular matrix synthesis. This suggested that CTGF may be an important therapeutic target for the control of connective tissue formation in fibrotic disorders. Recently, we have made a major breakthrough in our understanding of the molecular basis for CTGF mediation of TGF- $\beta$  mitogenic and matrigenic actions on connective tissue cells. We have determined that the individual domains of CTGF are responsible for signaling either the mitogenic activity (C-terminal domain of CTGF) or matrigenic activity (N-terminal domain). Additionally, we have found that two known growth factors function as essential co-factors for CTGF induction of DNA or collagen synthesis. Insulin-like growth factor 2 (IGF-2) is required for the induction of collagen synthesis and epidermal growth factor (EGF) is required for the induction of DNA synthesis. These findings suggest a common mechanism may exist for growth factors from other gene families to synergize with members of the CCN family to elicit biological responses in target cells, such as proliferation, differentiation and apoptosis.

#### A17 CTGF IN UTERINE FUNCTION

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Our interest in connective tissue growth factor (CTGF) arose through observations of the pig female reproductive tract in which we demonstrated a novel heparin-binding growth factor. This factor was subsequently shown to represent a highly truncated form of CTGF and to be one of several stable processed forms (102–260 residues) that are present in uterine luminal fluids (ULF) of pigs. We have subsequently shown that ULF contains proteases that rapidly convert 38 kDa CTGF to lower mass forms. In addition to its presence in the uterus of the pig, CTGF is also produced by the mouse and human uterus. As assessed by immunohistochemistry in these species, CTGF is localized primarily to uterine luminal and glandular epithelial cells during the estrous cycle and during the first few days of pregnancy. On the day of implantation in mice, epithelial staining for CTGF is strongly reduced and is followed over the next two days by profound staining of decidualizing endometrial stromal cells. The appearance of CTGF in differentiating endometrium, coupled with its ability to stimulate DNA synthesis, chemotaxis, cell proliferation and extracellular matrix production is consistent with a direct contribution of CTGF to the differentiation process.

Collectively, the data gathered so far suggest that CTGF is an important effector molecule in the mammalian uterus and that it plays a role in homeostatic mechanisms within normal uterine tissues during the estrous cycle and early pregnancy. The broad spectrum of biological activities of CTGF support its role in diverse processes within the uterine tract such as cell proliferation, differentiation, adhesion, chemotaxis, apoptosis and angiogenesis. Since most other published studies have focused on CTGF-related pathologies such as fibrosis, malignancy and wound healing, it is essential that a more thorough investigation of uterine CTGF be undertaken so that its role in normal tissue physiology be established. A central issue in CTGF biology remains its induction by transforming growth factor  $\beta$  (TGF- $\beta$ ) and whether, in fact, CTGF mediates some, and possibly many, of the activities that have previously been ascribed to TGF- $\beta$ . To start to address these issues, we have undertaken a detailed analysis of CTGF and TGF- $\beta$  at the utero-placental interface during early pregnancy in pigs, a species in which there is a high incidence of peri-implantation mortality, extensive remodelling of the extra-embryonic membranes, and loose diffuse non-invasive placentation. Our results show high correlation between TGF- $\beta$  and CTGF expression and describe CTGF mRNA and protein localization during the critical period of embryo attachment.

#### A18 TWISTED GASTRULATION IS A CONSERVED EXTRACELLULAR BMP ANTAGONIST

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Bone morphogenetic proteins (BMPs) are a subset of the transforming growth factor  $\beta$  (TGF $\beta$ ) super family of growth and differentiation factors that include the drosophila Decapentaplegic and related genes. BMP signaling regulates early dorsal-ventral cell fate decisions in flies, frogs and fish. The activity of these ligands in the extracellular space is regulated by several secreted factors including a secreted metalloprotease and several cysteine rich proteins such as the BMP antagonists Chordin and its drosophila homolog Short gastrulation (Sog). The *tsg* (twisted gastrulation) protein of drosophila is a second cysteine rich protein that is a member of the growing CCN superfamily of proteins. The functional properties of Tsg will be discussed. Engineered expression of *tsg* genes in Drosophila provides evidence for synergy with various other elements of the BMP signaling pathway. We find that Tsg enhances the antagonistic activity of Sog/Chordin towards BMP. In drosophila, visualization of BMP signaling activity using molecular markers reveals that the *tsg* loss-of-function phenotype is similar to that of *sog*. Studies with mouse, human and zebrafish homologs of Tsg demonstrate that Tsg molecules from different species are functionally equivalent. We conclude that Tsg is a conserved protein that functions together with Sog/Chordin to antagonize BMP signaling.

#### A19 INVOLVEMENT OF CYR61, AN ANGIOGENIC FACTOR, IN BREAST CANCER TUMOR PROGRESSION

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Cyr61, a member of a newly identified CCN gene family, was isolated and identified by differential expression between estrogen receptor (ER)-positive and ER-negative breast cancer cells. Cyr61 is a ligand for the integrin  $\alpha v \beta 3$ . We showed that expression of Cyr61 in HRG-transfected MCF-7 cells is greatly increased compared to parental MCF-7 cells and that it promotes invasion and metastasis. This phenotype is mediated, at least in part, by the regulation of MMPs and vascular endothelial growth factor (VEGF) in vivo. We also showed that Cyr61 is expressed in all the invasive, metastatic, heregulin (HRG) expressing, and ER-negative breast cancer cell lines. Moreover, Cyr61 was detected in about 30% of invasive human breast tumor biopsies. Most significantly, an anti-Cyr61 blocking antibody abolishes the invasiveness and migration of HRG-expressing breast cancer cells in vitro. Moreover, we have demonstrated that a functional blocking anti- $\alpha v \beta 3$  antibody blocks the Cyr61 induction of breast cancer cell invasion as measured by matrigel outgrowth. Recently, we demonstrated that Cyr61 is sufficient to confer estrogen independence and anti-estrogen resistance of breast cancer cells. In conclusion, these results strongly suggest that Cyr61 may play an important role in breast cancer progression possibly through regulation of invasion, metastasis, and neovascularization.

#### A20 CONTROL OF FIBROBLAST EXTRACELLULAR MATRIX PRODUCTION BY CTGF: REGULATION BY TGF $\beta$ , TNF- $\alpha$ AND IL-1

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**Background:** Scleroderma in connective tissue diseases is characterised by excessive synthesis and deposition of extracellular matrix components resulting in fibrosis/scarring of multiple organs, and ultimately leading to their dysfunction and failure. The growth factors transforming growth factor  $\beta$  (TGF $\beta$ ) and connective tissue growth factor (CTGF) are key effector molecules likely to be responsible for the persistent activation of the genes encoding extracellular matrix proteins, such as type I collagen, that underlie the fibrotic process.

Understanding the mode of action of these growth factors, in particular the pathways they activate, will allow the development of modulatory therapeutic strategies. We have studied the influence of the cytokines interleukin 1 (IL-1) and tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ) on the TGF $\beta$  induction of CTGF and type I collagen. Using a combination of signalling inhibitors and gene promoter reporter constructions, we have begun to investigate the mechanism by which these cytokines regulate TGF $\beta$  induced gene expression.

**Methods:** We have used primary dermal fibroblast cell cultures from normal individuals, enzyme linked immunosorbent assay (ELISA), western blotting and transient transfection using reporter gene constructs to examine the influence of IL-1 and TNF- $\alpha$  on the induction of fibroblast CTGF and type I collagen by TGF $\beta$ , and the effect of inhibiting 26S proteasome on matrix gene expression.

**Results:** TNF- $\alpha$  potently repressed TGF $\beta$  induction of CTGF and type I collagen expression. A role for the transcription factor NF $\kappa$ B in this down-regulation was suggested by the ability of inhibitors of the NF $\kappa$ B pathway/transport to block the effects of TNF- $\alpha$ . This was further supported by the ability of a dominant-negative mutant of I $\kappa$ B $\alpha$  to block the inhibition by TNF- $\alpha$ . Furthermore, we showed that addition of inhibitors of the 26S proteasome, MG-132 or lactacystin, which inhibit degradation of ubiquitinated proteins, prevented TNF- $\alpha$  inhibition of TGF $\beta$ -dependent CTGF and type I collagen expression.

**Conclusion:** TNF- $\alpha$  appears to be able to regulate CTGF and type I collagen expression via the NF $\kappa$ B pathway. In addition, the ubiquitin-dependent degradation of signalling molecules relevant to the TGF $\beta$  and the TNF- $\alpha$  (NF $\kappa$ B) pathway(s) is also likely to be a key regulatory event in controlling the expression of pro-fibrotic growth factors and matrix production.

#### A21 CYR61 PROMOTES THE SURVIVAL AND INHIBITS THE DIFFERENTIATION OF HEMATOPOIETIC STEM/PROGENITOR CELLS IN CULTURE

C-H. Yeung

Details not available.

#### A22 MOUSE NOV IS REGULATED BY PAX3 AND PROMOTES THE PROLIFERATION, SURVIVAL AND DIFFERENTIATION OF MYOBLASTS

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Nov is a member of an emerging family of genes (the CCN family) all of which encode regulators of cell growth. By mRNA in situ hybridization on mouse embryos (D Natarajan, *et al*, *Dev Dyn* [in press]) we have detected Nov transcripts in the lateral dermomyotome at 10.5 dpc and, later, in hypaxial muscles derived from non-migratory precursors. Interestingly, Pax3 becomes restricted to the lateral dermomyotome just prior to initiation of Nov expression in the same area and Nov transcripts were absent in hypaxial muscle precursor cells and hypaxial muscles of Sp/Sp embryos, which lack functional PAX3. PAX3 is involved in hypaxial muscle migration, differentiation and proliferation and has strong antiapoptotic activity (M Bernasconi *et al*, *Proc Natl Acad Sci U S A* 1996;93:13164-9; A-G Borycki *et al*, *Development* 1999;126:1665-74). By overexpressing full length (FLNOV) in embryonic stems (ES) cells we show that, in vitro, FLNOV increases cell proliferation and survival and promotes early myogenesis, but blocks terminal muscle differentiation. Furthermore, chimeric embryos from ES cell clones expressing FLNOV exhibit similar defects in muscle development. Muscles show higher cellularity, characterized by increased proliferation and survival, and histological abnormalities related to the inability of these cells to exit the cell cycle. Thus, we hypothesize that one of the mechanisms used by PAX3 to promote the development of the hypaxial muscles in vivo is to induce, directly or indirectly, Nov expression in muscle precursor cells.

#### A23 CHARACTERISATION OF XENOPUS LAEVIS CYR61

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Very little is known about the activities and roles of cyr61 in early embryonic development. To address this question, we used African clawed frog, *Xenopus laevis*, a well-suited system for studies of early

vertebrate development. We cloned the xenopus orthologue of cyr61, and determined its expression pattern. Abundant maternal transcripts of cyr61 are present until the end of cleavage stages of development (five hours post fertilisation). Zygotic transcription of cyr61 begins during neurula stages, with transcripts being localised to the somites. During tail bud stages, cyr61 transcripts are found in the notochord as well. At the tadpole stage, the developing heart begins to express cyr61. This expression pattern is reminiscent of the expression pattern of the mouse cyr61.

Misexpression of the full-length cyr61 leads to dorsalisation of the embryo; this effect appears, at least in part, to be due to the weak agonism with the Wnt pathway. However, in other assays, cyr61 can antagonise Wnt activity, suggesting that it may act as a context-dependent modulator of the Wnt pathway. We will present evidence that these two activities reside in different domains of cyr61.

These gain-of-function experiments describe a range of activities of cyr61 in the context of the early developing embryo. In order to uncover the role of cyr61 we are currently using recently developed antisense-based technology to deplete maternal cyr61 protein, and these results will be presented at the meeting.

#### A24 THE EXPRESSION PATTERN OF NOV-C DURING CHICK DEVELOPMENT AND ITS ROLE IN EMBRYOGENESIS

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Nov-C is a member of the CCN gene family which was identified as an associated gene to the chicken nephroblastoma induced by AMV transfection. We have done whole mount in situ hybridization to investigate its pattern of expression. In the early stage of development, HH stage 14, Nov-C was strongly expressed in the tail bud. At HH stage 20, its expression in the tail bud was reduced and became diffuse. On the contrary, expression in the central nervous system, the limb buds and the second pharyngeal arch is upregulated from this stage. The section of HH stage 20 showed its high expression in the dorsal part of hindbrain neuroepithelium and the distal area of mesenchyme in the limb buds and in the 2nd pharyngeal arch. We could not detect signal in mesonephros during embryogenesis. Nov-C expression seemed to be related to the development of the nervous system and the mesodermal tissues. We are trying to do forced expression of Nov-C in the neural tube by in ovo transfection to investigate its role in the nervous system and the neighboring tissues.

#### A25 THE ROLES OF CYR61 IN EMBRYONIC ANGIOGENESIS AND CHONDROGENESIS

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Expression of cyr61 is developmentally regulated in a tissue specific and temporally restricted manner. We have previously determined that the expression of cyr61 is closely linked to vascular and skeletal development during mouse embryogenesis, as revealed by in situ hybridization and immunohistochemistry. Purified Cyr61 protein stimulates directional migration of vascular endothelial cells through an integrin  $\alpha$ v $\beta$ 3-dependent pathway, and induces angiogenesis in a rat corneal micropocket implant assay. Furthermore, expression of cyr61 enhances tumorigenicity by increasing tumor size and vascularization in immunodeficient mice. Purified Cyr61 also promotes chondrogenic differentiation in mouse limb bud mesenchymal cells in micromass culture, and inhibition of endogenously expressed Cyr61 by neutralizing antibodies severely blunted chondrogenic differentiation. These results show that Cyr61 is both an angiogenic inducer and a chondrogenic differentiation factor, and may play roles in both angiogenesis and chondrogenesis during mammalian embryonic development. To investigate the developmental roles of Cyr61 further, we have generated mice deficient in Cyr61 by targeted gene disruption. Homozygous Cyr61<sup>-/-</sup> mutations lead to embryonic lethality due to multiple vascular defects. These results show that Cyr61 plays essential roles in vascular development during mouse embryonic development. Studies are under way to determine specific molecular and cellular defects associated with this phenotype.

**A26 CONNECTIVE TISSUE GROWTH FACTOR (CTGF) IS ESSENTIAL FOR SKELETAL DEVELOPMENT IN THE MOUSE**

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CTGF is a member of the CCN family of secreted proteins. Members of this family have been shown to have diverse effects on cell proliferation and differentiation in vitro, but their roles in vivo during development are not known. In order to identify potential cellular targets of CTGF activity, we used in situ hybridization to examine Ctgf expression in developing mouse embryos. These experiments showed high levels of Ctgf mRNA in proliferating cartilage and in vascular tissues. In addition, strong expression was seen at the osteogenic front in growth plates, suggesting potential roles in multiple aspects of chondrogenesis. In order to test the hypothesis that CTGF affects chondrocytic cell proliferation and/or differentiation, we used a retroviral construct to express CTGF in primary bone marrow stromal cells and in primary articular chondrocytes. These experiments demonstrated that CTGF overexpression affected cell proliferation and collagen synthesis. Moreover, proliferation and survival of primary hypertrophic chondrocytes was affected by high levels of CTGF expression. Therefore, CTGF may play multiple roles during chondrogenesis. To test the hypothesis that CTGF is required for skeletal development, we generated Ctgf<sup>-/-</sup> mice using gene targeting techniques. Mice lacking CTGF die immediately after birth as a result of respiratory distress, which is a secondary consequence of multiple skeletal defects. Therefore, Ctgf is required for skeletal development in the mouse. Histological and molecular marker studies are in progress to define the cellular basis for this phenotype.

**A27 EFFECTS OF CTGF/HCS24, A PRODUCT OF A HYPERTROPHIC CHONDROCYTE-SPECIFIC GENE, ON THE PROLIFERATION AND DIFFERENTIATION OF CHONDROCYTES AND OSTEOBLASTS IN CULTURE**

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Recently, we cloned an mRNA predominantly expressed in hypertrophic chondrocytes from a human chondrosarcoma-derived chondrocytic cell line, HCS-2/8, by differential display-polymerase chain reaction (PCR) and found that its gene, named hcs24, was identical with that of connective tissue growth factor (CTGF). Here we investigated CTGF/Hcs24 function in chondrocytes and osteoblasts in culture. The recombinant CTGF/Hcs24 (rCTGF/Hcs24) effectively promoted the proliferation of HCS-2/8 cells and rabbit growth cartilage (RGC) cells in a dose dependent manner and also increased dose-dependently the proteoglycan synthesis in these cells. In addition, these stimulatory effects of the CTGF/Hcs24 were neutralized by the addition of anti-CTGF antibodies. Furthermore, the rCTGF/Hcs24 effectively increased the alkaline phosphatase activity in RGC cells in culture. Moreover, reverse transcription (RT)-PCR analysis revealed that the rCTGF/Hcs24 stimulated gene expression of aggrecan and collagen types II and X in RGC cells in culture. These results indicate that CTGF/Hcs24 directly promotes the proliferation and differentiation of chondrocytes.

rCTGF/Hcs24 also promoted the proliferation of Saos-2 cells and a mouse osteoblast cell line MC3T3-E1 in a dose- and time-dependent manner. rCTGF/Hcs24 also increased mRNA expression of type I collagen, alkaline phosphatase, osteopontin and osteocalcin in both Saos-2 cells and MC3T3-E1 cells. Moreover, rCTGF/Hcs24 increased alkaline phosphatase activity in both cells. It also stimulated collagen synthesis in MC3T3-E1 cells.

Furthermore, rCTGF/Hcs24 stimulated the matrix mineralization on MC3T3-E1 cells and its stimulatory effect was comparable to that of bone morphogenetic protein-2. These findings indicate that CTGF/Hcs24 is a novel, potent stimulator for the proliferation and differentiation of osteoblasts in addition to chondrocytes and endothelial cells (T Shimo *et al.* *J Biochem (Tokyo)* 1998;124:130-40, and *J Biochem (Tokyo)* 1999;126:137-45). Because of these functions, we are re-defining CTGF/Hcs24 as a major factor to promote endochondral ossification to be called "ecogenin: endochondral ossification genetic factor".

**A28 CCN FAMILY MEMBERS IN URODELE LIMB REGENERATION**

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Amphibian limb regeneration constitutes an interesting model for studying morphogenesis, cell-cell interactions, signalling and spatial patterns of cell differentiation which are specified according to positional identity. Limb regeneration is distinct from cellular turnover and from the regulation of embryonic structures at stages before commitment and differentiation. It remains a challenge, however, to understand precisely how the combination of tissue repair mechanisms with reactivation of embryonic programs can generate growth, pattern formation, and morphogenesis in an adult animal.

Amputation of the limb in a larval or adult urodele such as the newt, is followed by rapid migration of epithelial cells which form the wound epithelium. In response to amputation or tissue removal, local reversals in the differentiated state of cells are observed and the post-mitotic cells of the limb mesenchyme beneath the wound epidermis re-enter the cell cycle. Connective tissue fibroblasts, cartilage and muscle contribute to this growth zone. These resulting cells, called blastemal cells, express several markers that are not expressed by differentiated mesenchymal cells of the normal limb nor by cells of the developing limb bud. The blastemal cells then re-differentiate in a proximal to distal sequence to restore the missing limb parts (JP Brockes, *Science* 1997;276:81-7).

Positional memory is respecified in a graded and dose-dependent fashion by retinoic acid (RA) and precursor retinoids which proximalize the blastema. Thus, a wrist blastema when treated with RA will regenerate an arm which has a normal structure, but arises from an inappropriate proximodistal level. Taking advantage of the proximalization of blastemal cells by RA (LT Pecorino *et al.* *Curr Biol* 1996;6:563-9), subtraction libraries have been obtained from blastemas treated with RA and with the RA solvent DMSO. A total of approximately 1500 clones have been analysed, 80% of which are unknown genes. Newt CTGF has been identified as an RA-regulated gene in newt blastemal cells (DE Cash *et al.* *Gene* 1998;222:119-24). It is apparently expressed in the basal layers of the wound epidermis and the mesenchymal blastema. We are particularly interested in cell-surface proteins which might mediate cell-cell interactions responsible for positional identity. Expression patterns and functional studies will be presented for a novel protein with the characteristic pattern of Cys residues shown by molecules such as CTGF, CD59 complement regulator and xenoxin.

**A29 CTGF AND EXTRACELLULAR MATRIX REMODELING: IMPLICATIONS FOR DIABETIC NEPHROPATHY**

J. Crean

Absent.

**A30 URINARY CTGF: A POTENTIAL PREDICTOR OF DIABETIC NEPHROPATHY**

B. L. Riser, P. Cortes, J. Grondin, P. V. Deshmukh, P. S. Chahal. Division of Nephrology, Henry Ford Health System, Detroit MI, USA

It is currently impossible to predict reliably which diabetic patients will develop diabetic nephropathy (DN) and progress to kidney failure. As a result, there is hesitation to begin lifelong treatment that may retard progression. Microalbuminuria, often taken as a predictor of overt DN is, in fact, an indicator of established glomerular damage. We have recently shown that CTGF expression is upregulated more than 25-fold in the glomerulus during early experimental diabetes. Further, the addition of connective tissue growth factor (CTGF) to mesangial cell cultures increases their production of extracellular matrix (*J Am Soc Nephrol* 2000;11:25-38). Here, we investigated whether CTGF is present in the urine and its relationship to diabetes or DN. An enzyme linked immunosorbent assay was used to determine CTGF levels in control and STZ-diabetic Munich-Wistar rats. Barely detectable levels of CTGF were present in control animals (0.97 ± 0.22 ng/μg creatinine; n = 12), but increased approximately 15-fold after one month (14.6 ± 2.8 ng/μg creatinine; n = 4) and remained elevated after nine months of diabetes (9.61 ± 2.04 ng/μg

creatinine;  $n = 6$ ). Studies were also carried out on healthy volunteers, patients with diabetes but without current evidence of nephropathy, or with nephropathy. Low levels of CTGF were present in the healthy volunteers ( $0.69 \pm 0.10$  ng/ $\mu$ g creatinine;  $n = 4$ ), but increased fourfold in those with nephropathy ( $2.79 \pm 0.83$  ng/ $\mu$ g creatinine;  $n = 6$ ). Half of those with diabetes but without nephropathy had significantly elevated CTGF levels ( $5.55 \pm 0.08$  ng/ $\mu$ g creatinine  $n = 3$ ), whereas the other half had levels ( $0.57 \pm 0.08$  ng/ $\mu$ g creatinine) equal to those of healthy volunteers. Western blotting of healthy volunteer samples identified a single band that migrated in parallel with recombinant CTGF. In contrast, those with DN demonstrated multiple CTGF bands. These findings support our hypothesis that CTGF is upregulated early during the evolution of DN. We allege that increasing urinary CTGF may predict those patients destined for progressive nephropathy and end-stage disease.

### A30 KINETICS OF CONNECTIVE TISSUE GROWTH FACTOR EXPRESSION DURING EXPERIMENTAL PROLIFERATIVE GLOMERULONEPHRITIS

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Connective tissue growth factor (CTGF) is a member of the CCN family of immediate early genes, which are involved in cell proliferation, migration and matrix production. Recently, CTGF was observed to be strongly upregulated in human proliferative and fibrogenic renal disease. By in situ hybridisation and reverse transcription polymerase chain reaction, the expression of CTGF was investigated in experimental proliferative glomerulonephritis induced by injection of anti-Thy-1.1 antibody in the rat. CTGF expression in cultured rat mesangial cells and glomerular visceral epithelial cells (GVEC) was studied in response to transforming growth factor  $\beta$  (TGF- $\beta$ ), an essential pathogenetic factor in this model. In normal rat kidneys, only some GVEC expressed CTGF mRNA. In anti-Thy-1.1 nephritis, CTGF mRNA expression was strongly increased in extracapillary and mesangial proliferative lesions and in areas of periglomerular fibrosis. Early glomerular CTGF overexpression in GVEC coincided with a striking upregulation of TGF- $\beta$ 2 and to a lesser extent of TGF- $\beta$ 3. Glomerular CTGF mRNA expression was maximal at day 7, in association with increased TGF- $\beta$ 1 mRNA and protein expression. CTGF mRNA overexpression by parietal epithelial cells preceded the periglomerular appearance of  $\alpha$  smooth muscle actin positive fibroblasts. In cultured mesangial cells, TGF- $\beta$ 1, TGF- $\beta$ 2 and TGF- $\beta$ 3 transiently increased the CTGF/glyceraldehyde-6-phosphate dehydrogenase (GAPDH) mRNA ratio upto threefold compared with controls at four hours. In GVEC, upregulation of CTGF mRNA by these TGF- $\beta$  isoforms was more sustained, being eight to 16 fold versus controls at 24 hours. The kinetics of CTGF expression strongly suggest a role in glomerular repair, possibly downstream of TGF- $\beta$ , in this model of transient renal injury.

### A32 CONNECTIVE TISSUE GROWTH FACTOR (CTGF) EXPRESSION IN EXPERIMENTAL AND HUMAN LIVER FIBROGENESIS

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Connective tissue growth factor (CTGF) is a 38 kDa, cystein-rich, mitogenic peptide that is selectively induced in fibroblasts by transforming growth factor  $\beta$  (TGF- $\beta$ ). It stimulates in vitro fibroblast proliferation and extracellular matrix synthesis. To assess the role of CTGF in liver fibrogenesis, we investigated CTGF mRNA and protein expression by immunohistochemistry, western-blot and in situ hybridization with either CTGF specific polyclonal antibody or digoxigenin-labelled riboprobe in a model of rat fibrosis and in human liver biopsies.

In the rat model of CCl<sub>4</sub>-induced liver fibrosis, no or mild staining was observed in early sacrificed rat livers whereas CTGF was observed later on with the development of septal fibrosis and cirrhosis. CTGF expression was located in areas of fibrogenesis and intensity of staining was correlated with the degree of fibrosis ( $\chi^2 = 5.6$ ,  $p < 0.02$ ) but not with necroinflammatory lesions.

To assess the expression of CTGF in humans, 39 liver biopsies of patients with chronic hepatitis C (group 1) and 28 biopsies with other chronic liver diseases (group 2) were investigated. In group 1, a moderate or strong CTGF immunostaining was observed in 21 out of 39 cases at the interface between portal tracts and lobules and, in 11 cases, in the sinusoidal lining around portal tracts. Semiquantitative score of CTGF immunostaining was correlated with the score of fibrosis ( $\chi^2 = 7.9$ ,  $p < 0.02$ ) but not with the grade of activity. Furthermore, a correlation was observed between CTGF immunostaining and activation of hepatic stellate cells (HSC) as assessed by  $\alpha$  smooth muscle actin immunostaining ( $\chi^2 = 18.7$ ,  $p < 0.05$ ). In situ hybridization showed CTGF mRNA expression in spindle cells both in fibrous septa and sinusoidal lining. In group 2, CTGF immunostaining was moderate or strong in 21 out of 28 cases, regardless of the etiology of the liver disease. Again, a significant correlation was observed between extent of fibrosis and CTGF expression ( $\chi^2 = 8.2$ ,  $p < 0.02$ ). Finally when CTGF expression was studied by western-blot in cultured HSC lysates, a strong 38 kDa band was observed.

In conclusion, this study shows that CTGF is strongly expressed during liver fibrogenesis. HSC seems a major source of CTGF in the liver.

### A33 REGULATION AND FUNCTION OF CTGF IN RENAL FIBROSIS: IMPLICATIONS FOR DIAGNOSIS AND CONTROL OF DISEASE PROGRESSION

B. L. Riser, P. Cortes, J. Yee, J. M. Grondin. Department of Internal Medicine, Henry Ford Hospital, Detroit, Michigan, USA

Connective tissue growth factor (CTGF) is a profibrotic factor and is upregulated in many cell types by transforming growth factor  $\beta$  (TGF- $\beta$ ). The long-term overexpression of TGF- $\beta$  in glomerular mesangial cells is a primary causal factor for mesangial matrix expansion, progressive sclerosis and end-stage renal disease. Therefore, we investigated the possibility that CTGF might be a downstream mediator of renal sclerosis. To accomplish this, cultured rat mesangial cells (MC), renal cortex, and microdissected glomeruli from mice with diabetes, a prototype for progressive renal fibrosis, were studied. In culture, unstimulated MC expressed low levels of CTGF message and secreted minimal amounts of full-length CTGF protein. However, exposure to TGF- $\beta$ , high glucose concentrations, or cyclic stretch, all causal factors in glomerulosclerosis, markedly induced the expression of CTGF transcripts. With all but stretch there was a concomitant stimulation of CTGF protein secretion. TGF- $\beta$  also induced abundant quantities of a small molecular weight form of CTGF (18 kDa). The induction of CTGF protein by a high glucose concentration was mediated by TGF- $\beta$ , since a TGF- $\beta$  neutralizing antibody blocked this stimulation. The relevance of elevated CTGF was demonstrated by the ability of the recombinant CTGF to increase MC production of fibronectin and collagen type I, two key mesangial matrix proteins. In normal, control db/m mice, glomerular CTGF mRNA levels were low. By contrast, after 3.5 months of diabetes, the obese db/db mice demonstrated a 27-fold increase in expression. This upregulation represented an early response since mesangial expansion was mild, and interstitial disease and proteinuria were not yet apparent. The primary alteration of CTGF appeared to reside in the glomerulus since whole kidney cortices increased only twofold with diabetic disease. These results provide evidence for the role of CTGF in the pathogenesis of mesangial matrix accumulation and glomerulosclerosis. Since increased MC cyclic stretch from elevated glomerular pressure, hyperglycemia, and increased TGF- $\beta$  activity all play a role glomerular injury and subsequent progressive glomerulosclerosis, CTGF is likely to be a suitable target for both the prevention of renal damage and the diagnosis of early disease.

### A34 NOVH EXPRESSION IN MUSCULOSKELETAL TUMORS

M. C. Manara

Details not available.

### A35 CCN-FAMILY MEMBERS IN PANCREATIC DISEASES

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In a previous study connective tissue growth factor (CTGF) was identified as target gene of transforming growth factor  $\beta$  (TGF $\beta$ ) and TGF $\alpha$  in pancreatic cancer cells. In the present study we investigated

the expression of the CCN-family members CTGF, Cyr61 and nov in pancreatic cancer, chronic pancreatitis and in normal pancreatic tissue samples. Both, CTGF and cyr61 mRNAs are moderately overexpressed in chronic pancreatitis and highly overexpressed in pancreatic cancer as compared to normal pancreatic tissue. Nov mRNA is moderately overexpressed in pancreatic cancer. By in situ hybridization in pancreatic cancer tissues and analysis of nude mice xenografts we could show that stromal cells are the predominant site of CTGF and Cyr61 expression in pancreatic tumors. CTGF and Cyr61 are also expressed in pancreatic cancer cell lines at varying levels and their expression in Panc1 cells is inducible by epidermal growth factor (EGF) and TGF $\alpha$  in an early and transient fashion, whereas TGF $\beta$  induces a prolonged expression.

Since CTGF and Cyr61 are known to induce proliferation and extracellular matrix production in fibroblasts we suggest that these growth factors may participate in the development of the marked desmoplastic reaction which is characteristic for pancreatic cancer. In the same way CTGF and Cyr61 may be involved in the development of fibrosis and inflammation in chronic pancreatitis.

### A36 PATHOGENESIS OF SCLERODERMA

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Scleroderma (systemic sclerosis: SSc) is a multi system disorder of connective tissue characterized by excessive fibrosis in the skin and various internal organs. Although the pathogenesis of this disease remains unknown, growth factors and cytokines that are released from inflammatory cells infiltrating affected tissues have been suggested to play a central role in initiating and developing fibrosis in SSc. Transforming growth factor- $\beta$  (TGF- $\beta$ ) is likely to be one of the most significant candidates responsible for fibrosis in SSc.

TGF- $\beta$  is shown to have indirect mitogenic activity on fibroblasts, and this mitogenic activity appears to be dependent on the autocrine production of platelet-derived growth factor (PDGF)-related peptide, which was identified as connective tissue growth factor (CTGF). CTGF is selectively induced in fibroblasts after activation with TGF- $\beta$ . Thus, CTGF functions as a downstream mediator of TGF- $\beta$  action on connective tissue cells, where it stimulates cell proliferation and extracellular matrix synthesis. CTGF has been suggested to be involved in development of fibrosis in SSc since CTGF mRNA expression is upregulated in the sclerotic skin from patients with SSc.

Recently, we examined the serum concentration of CTGF in patients with SSc. The serum levels of CTGF were increased in patients with SSc when compared with normal controls. Furthermore, the elevated CTGF levels correlated with the extent of skin sclerosis and the severity of pulmonary fibrosis.

Next, to establish an appropriate animal model of skin fibrosis by exogenous application of growth factors, we investigated the in vivo effects of growth factors by injecting TGF- $\beta$ , CTGF and basic fibroblast growth factor (bFGF) into the subcutaneous tissue of newborn mice. A single application of TGF- $\beta$  or bFGF resulted in the formation of transient granulated tissue that disappeared despite seven days of consecutive injections. However, injecting TGF- $\beta$  plus CTGF produced long-term fibrotic tissue, which persisted for at least 14 days.

Also, fibrotic tissue was observed when CTGF was injected from four to seven days after TGF- $\beta$  injections for the first one to three days.

From the data of TGF- $\beta$  and CTGF in various skin fibrotic disorders described above, I would like to propose a "two step fibrosis hypothesis in systemic sclerosis".

I believe TGF- $\beta$  induces first fibrosis in the early stage of SSc and CTGF acts to maintain tissue fibrosis in the whole stage.

### A37 EXPRESSION PATTERNS OF NOVH IN NORMAL AND TUMOR TISSUES

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The novH protein is a secreted 46 kDa cysteine-rich glycoprotein, identified by sequence homologies as a member of the newly described CCN family (CTGF, CYR61, NOV) of growth related genes. We have previously reported that novH is expressed in a restricted pattern in developing kidney. Comparable and even higher levels of expression were found in the blastema and heterotypic differentiated elements in Wilms tumors.

Because novH is a secreted protein, delineating its expression patterns in tissues at the cellular level requires detection by both immunohistochemical methods and in situ hybridization. We will present our experience using immunohistochemistry with antigen retrieval and non-isotopic in situ hybridization in the characterization of novH expression in fetal and adult tissues as well as in a variety of tumors. Apart from antibody concentration and incubation parameters, other methodological variables like antigen retrieval buffer pH were found to determine the ultimate strength and specificity of the detection signal. Further results will be presented on expression of novH in cultured cells using immunofluorescence based detection methods. Overall, our observations suggest that novH plays a role in the biology of a diversity of cell types, and that its expression is associated with functional differentiation in a developmentally regulated manner.

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## Correction

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In the letter entitled "Formic acid decalcification of bone marrow trephines degrades DNA: alternative use of EDTA allows the amplification and sequencing of relatively long PCR products" (December 2000, vol 53, page 336) the authors were listed in the wrong order. They should have been in the following order: Wickham CL, Sarsfield P, Joyner MV, Jones DB, Ellard S, Wilkins B. The journal apologises for any inconvenience this may have caused.



## Formic acid decalcification of bone marrow trephines degrades DNA: alternative use of EDTA allows the amplification and sequencing of relatively long PCR products

P Sarsfield, C L Wickham, M V Joyner, et al.

*Mol Path* 2000 53: 336

doi: 10.1136/mp.53.6.336

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