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ABSTRACTS

Fifth International Workshop on the CCN Family of Genes: Abstracts and Posters October 18–22, 2008

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MATRICELLULAR PROTEINS REGULATE CELL FUNCTION: STUDIES OF THROMBOSPONDINS 1 AND 2

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Matricellular proteins are present in the extracellular space, but do not perform structural functions. Rather, they modulate cell-matrix interactions and cell function by interacting with a wide variety of cell-surface receptors, cytokines and growth factors. The term 'dynamic reciprocity' has been applied to this process. Thrombospondins (TSPs) 1 and 2 resemble each other structurally and have similar properties when used as purified proteins in assays in vitro. However, physiologically they perform different functions, as judged by the phenotypes of TSP1 and 2-null mice, and by the fact that they do not compensate for each other in vivo. The latter finding can be explained by the very different promoter sequences in the *thbs1* and *thbs2* genes, which dictate different temporal and spatial expression of the two proteins in animals.

This presentation will focus on the phenotype of the TSP2-null mouse, as elucidated by studies conducted in my laboratory during the past 10 years. In particular, I will emphasize the importance of a homeostatic function for both TSP1 and TSP2 in the inhibition of angiogenesis. This function, in contrast to the ligation of the CD36 receptor, which reduces vascularity by causing apoptosis of endothelial cells (EC), inhibits EC proliferation by a pathway that is initiated by interaction of TSPs with the very low density lipoprotein (VLDL) receptor. In effect, one can thus 'slow a car down by braking rather than by driving it over a cliff'

THE CCN FAMILY OF PROTEINS: STRUCTURE-FUNCTION RELATIONSHIPS

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The CCN proteins are key signalling and regulatory molecules involved in many vital biological functions including cell proliferation, angiogenesis, tumourigenesis and wound healing. How these proteins influence such a range of functions remains incompletely understood, but is likely related to their discrete modular nature and a complex array of intra–and inter-molecular interactions with a variety of regulatory proteins and ligands. Although certain aspects of their biology can be attributed to the four individual modules that constitute the CCN proteins, recent results suggest that some of their biological functions require co-operation between modules. Indeed, the modular structure of CCN proteins provides important insight into their structure-function relationships.

NUCLEOPHOSMIN/B23: A MULTIFUNCTIONAL REGULATOR THAT DETERMINES THE FATE OF CCN2 mRNA

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CCN2/CTGF is a multifunctional molecule that has been known to play a central role in chondrocyte differentiation. During this process, the expression of *ccn2* is tightly regulated to confer a maximal level at prehypertrophic—hypertrophic stages, in which the 3'-untranslated region (UTR) of the mRNA is critically involved in mediating its post-transcriptional regulation. In our previous studies, we found that a 40 kDa protein binding specifically to an RNA *cis*-element, 3'-100/50, in the 3'-UTR of the chicken *ccn2* mRNA regulated its intracellular stability. Interaction of the 40 kDa protein and 3'-100/50 is enhanced in proliferating chondrocytes, in which *ccn2* mRNA is rapidly degraded; whereas prolonged half life of *ccn2* mRNA is observed in hypertrophic chondrocytes, where the interaction of the 40 kDa-protein and 3'-100/50 is diminished. Collectively, the 40 kDa protein has been thought to be a *ccn2*-specific mRNA destabilizer during chondrocyte differentiation.

Here, we finally identified this 40 kDa protein as nucleophosmin (NPM)/B23. NPM is a nuclear-cytoplasmic shuttling protein that is characterized by its multiple functionality. This protein has been known as a histone chaperone, regulator of ribosomal RNA transcription, as well as an RNA binding post-transcriptional regulator of gene expression. In our hands, direct binding to NPM to 3'-100/50 was confirmed not only by RNA EMSA and UV crosslinking assay, but also by RNA immunoprecipitation analysis. By using recombinant chicken NPM, we could successfully reconstitute the post-transcriptional regulation of *ccn2* by NPM *in vitro* and found that this regulation was more robust in chondrocytes than fibroblasts. Furthermore, siRNA-mediated gene silencing of NPM *in vivo* clearly showed enhanced *ccn2* gene expression and prolonged half life of the *ccn2* mRNA, confirming the functional property of NPM as a specific destabilizer of the *ccn2* mRNA in living cells.

The 5'-100/50 element, a target of NPM, is evolutionally conserved among vertebrate species. Therefore, it is anticipated that NPM is a critical regulator of CCN2 during endochondral ossification and possibly, in other physiological and pathological states as well in

mammals. To confirm/uncover these aspects, further investigation with mammalian species is currently in progress.

REGULATION OF CHONDROCYTIC PHENOTYPE BY MICRO RNA 18A: INVOLVEMENT OF *CCN/CTGF* AS A MAJOR TARGET GENE

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Micro RNA (miRNA) is a major class of non-coding RNAs that are involved in a variety of biological events including development of a number of tissues and organs in higher eukaryotes. In order to identify miRNAs that regulate endochondral ossification, we searched for miRNA candidates that were down-regulated in chondrocytic cells and were predicted to target CCN2 family protein 2/connective tissue growth factor (CCN2/CTGF), which has been known to promote endochondral ossification and cartilage regeneration, by a combination of microarray and *in silico* analyses. Five miRNAs were predicted to target the *Ccn2* 3'-UTR. Among those candidates, expression of miR-18a was found to be the most strongly repressed in chondrocytic cells. Utilizing reporter gene constructs and a synthetic mature miR-18a duplex, we experimentally confirmed an miR-18a target in the same region in the 3'-untranslated region (UTR) of human *Ccn2* as predicted *in silico*. Also, the introduction of the miR-18a duplex efficiently repressed the production of CCN2 in those cells. Interestingly, this *Ccn2* silencing was conferred entirely at a translation stage without affecting the steady-state mRNA level in chondrocytic HCS-2/8 cells; whereas accelerated degradation of *Ccn2* mRNA has been observed in human breast cancer MDA-231 cells. Finally, transfected miR-18a duplex significantly caused the repression of the mature chondrocytic phenotype. Our present study revealed a regulatory role for miR-18a in chondrocytic differentiation through CCN2 and a variable mode of post-transcriptional regulation of the same miRNA, which was dependent on the cellular background.

CTGF/CCN-2 EXPRESSION INDUCED BY TGF-b IS MODULATED THROUGH A MECHANISM DEPENDENT OF DECORIN AND LRP-1

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Duchenne Muscular dystrophy (DMD) is the most severe myopathy characterized by degeneration of skeletal muscle fibers and its replacement by connective tissue producing fibrosis. Connective tissue growth factor (CTGF/CCN-2), one of the main inducers of fibrosis is increased in skeletal muscle of patients with DMD. Diverse extracellular growth factors have been proposed to modulate the levels of CTGF, regulating the beginning and progression of fibrosis. Among them we can find transforming growth factor beta (TGF-b) which is augmented in skeletal muscle of DMD. In skeletal muscle cells, we have demonstrated that CTGF expression is increased by TGF-b. A regulator of TGF-b activity is decorin, a soluble proteoglycan present is skeletal muscle which is endocytosed through its receptor LDL-related prtein-1 (LRP-1). We have previously demonstrated that decorin modulates the response of myoblasts to TGF-b.

In this study, we evaluated the participation of decorin and its receptor LRP-1 in the regulation of CTGF expression induced by TGF-b in skeletal muscle cells. TGF-b increased CTGF expression in a concentration dependent fashion. Transfection experiments using a plasmid reporter for CTGF transcriptional activity (pCTGF-luc) showed that myoblasts that not express decorin (Dcn null) have 50% less response to TGF-b compared to wild type myoblasts. This effect was rescued to the wild type levels when Dcn null cells re-express decorin. To evaluate if the effect of decorin in CTGF expression induced by TGF-b was mediated by LRP-1, myoblasts wild type and Dcn null were co-transfected with a specific siRNA for LRP-1 and the plasmid reporter pCTGF-luc. Under these conditions, pCTGF-luc activity induced by TGF-b decreased only in cells expressing decorin, but it was unchanged in Dcn null cells. The same results were obtained when mRNA levels of CTGF induced by TGF-b were evaluated. These results suggest that decorin and LRP-1 modulate the TGF-b-dependent expression of CTGF. Interestingly, wild type and Dcn null cells incubated with a specific inhibitor of TGF-b receptor I kinase activity (SB 431542) or with a siRNA for Smad-2/3, showed that CTGF expression induced by TGF-b decreased to basal levels, indicating that Smad-pathway was essential to TGF-b dependent expression of CTGF.

These studies suggest that CTGF expression induced by TGF-b is modulated through a decorin dependent mechanism involving its endocytic receptor LRP-1.

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IDENTIFICATION OF SOX9 BINDING SITE IN CCN2 (CTGF) GENE BY USE OF ChIP ON CHIP ANALYSIS

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The transcription factor Sox9 is essential for several steps of the chondrocyte differentiation pathway including chondrogenic mesenchymal condensation. To determine which genes upregulated during chondrocyte differentiation are direct targets of Sox9, and to find out the Sox9 binding site in the genes, we have performed chromatin immunoprecipitation (ChIP) of chondrocytes using Sox9 antibodies. The immunoprecipitated DNA was amplified and hybridized to a custom-made high-density microarray, which covered a total 93 genes from 15-kb 5' to 10-kb 3' of these genes with 50-mer oligonucleotides containing 20 nucleotides overlap between oligos (ChIP on chip).

Several lines of evidence indicate that CCN2 (CTGF) enhances the proliferation and maturation of chondrocytes. Therefore we added CCN2 gene in our ChIP on chip analyses supposing this might be a direct target of Sox9. In the CCN2 gene, only one high affinity site was detected in its promoter region by anti-Sox9- ChIP on chip, but not by conrtol IgG ChIP on chip. This site was detected in ChIP on chip analyses using both sense–and anti-sense DNA as probe. In order to verify this Sox9 binding site, we prepared several reporter constructs which have different length of promoter region of CCN2. The sequence between −610 and −500 upstream from transcription start site is required for Sox9 to enhance the promoter activity in a reporter assay. Further, the Sox9 binding site has been confirmed by EMSA assay and ChIP experiment. These results suggest that CCN2 (CTGF) is a direct target of Sox9 in chondrocytes and should be regulated by it during chondrogenesis.

MECHANICAL REGULATION OF THE CYR61/CCN1 GENE REQUIRES THE COMBINED ACTIVITY OF THE MYOCARDIN-RELATED TRANSCRIPTION FACTOR (MRTF)—A AND P300/CBP IN SMOOTH MUSCLE CELLS

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Mechanical regulation of the Cyr61 gene involves signaling through RhoA GTPase-dependent actin polymerization which essentially controls the activation of transcription factors such as SRF. SRF activity requires the relocalization of the SRF co-activator MRTF-A from the cytoplasm to the nucleus and tethering SRF to CArG box sequences. We hypothesized that both SRF and MRTF-A mediate mechanical strain-induced Cyr61 gene expression.

The activity of a reporter construct containing 2,395 nucleotides upstream the initiation start site of the human Cyr61 gene was increased (up to 39-fold) by continuous cyclic stretching of smooth muscle cells (SMCs). When the CArG box sequence for SRF binding was mutated (CCAAA ➔ AGATC), the promoter activity was significantly reduced (5.6-fold increase only) in mechanically stimulated cells. Similarly, cotransfection with plasmid vectors expressing dominant negative forms for either SRF or MRTF-A but not MRTF-B, a structurally-related form of MRTF-A, significantly decreased the Cyr61 promoter activity. The activity of the Cyr61 promoter reporter was significantly reduced in SMCs isolated from MRTF-A -/- mice in response to mechanical strain. Cells cultured from MRTF-A -/- showed reduced adhesion points and stress fibers compared to cells from wild type mice which is consistent with the important role of MRTF-A in muscle fiber growth and maturation. MRTF-A, which was shown to bind to unpolymerized actin (G-actin), was localized within the cytoplasm in unstimulated cells and accumulate within the nucleus in mechanically stimulated cells. Consistent with these results, preincubation of the cells with inhibitors of actin polymerization such as latrunculin B, or NTSMA, a cell-penetrating peptide containing the N-terminal sequence Ac-EEED of smooth muscle -actin, that interfere with actin polymerization, suppressed both nuclear translocation of MRTF-A and Cyr61 promoter activation by cyclic stretch. Leptomycin A, an inhibitor of nuclear export, induced nuclear accumulation of MRTF-A but did not increase the Cyr61 promoter reporter gene activity in unstimulated cells suggesting that (i) MRTF-A continuously shuttles between cytoplasm and nucleus and (ii), nuclear localization of MRTF-A was not sufficient to activate the Cyr61 promoter. Interestingly, Cyr61 promoter activity was completely abolished by curcumin, a pharmacological inhibitor of p300/histone acetylase activity. Expression of p300 alone was insufficient to activate the Cyr61 promoter reporter but augmented the promoter activity in the presence of leptomycin A. Co-immunoprecipitation studies demonstrated direct interaction between p300 and MRTF-A in mechanically stimulated cells. These studies reveal that the combined activities of MRTF-A and p300 are involved in the mechanical regulation of the Cyr61 gene.

PERIOSTIN IS EXPRESSED IN HUMAN SKIN AND IS REGULATED BY MECHANICAL STRAIN

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Periostin is a novel secreted matricellular protein with very diverse functions that appear necessary for postnatal development in collagen-rich tissues. The expression of periostin is most common in collagen-rich connective tissues that are continually subject to mechanical strains as a result of normal tissue function. We have recently identified the presence of periostin protein in human skin, with the protein localizing to the basement membrane, keratinocytes and dermal fibroblasts. Furthermore, the skin of periostin knockout mice is significantly thinner than their wild type counterparts, has reduced collagen fibrillogenesis and cross-linking, which correlates with reduced compliance in comparison with wild type mice. We hypothesize that periostin is required for normal tissue homeostasis, and will play a major role in cutaneous wound repair. To investigate the control of periostin expression, fibroblasts were cultured on collagen I under static conditions or 10% cyclic strain. Periostin mRNA levels increased under cyclic loading, and showed a similar expression pattern to thrombospondin-1 and collagen I. To assess if culture matrix was a determinant of periostin expression, fibroblasts were cultured under the same strain conditions, except on collagen IV. Periostin expression increased 2 fold higher in fibroblasts on collagen IV compared to those cultured on collagen I. To further examine periostin expression, we utilized a punch wound cutaneous model in C57Bl/6 mice. Wounds were assessed at 3, 7, and 21 days using immunohistochemistry. Periostin expression was evident in granulation tissue by day 3, and levels peaked at day 7. By day 21, periostin expression declined to basal levels. We conclude that periostin expression in normal skin may be regulated by mechanical strain, and that periostin up-regulation in cutaneous wound repair is required for remodeling of the extracellular matrix. Future experiments will focus on assessing wound repair in periostin knockout mice, as well as the "outside-in" signaling controlling periostin expression.

FUNCTIONAL AND BIOCHEMICAL ASSESSMENT OF CCN5 TARGETED TO THE NUCLEUS

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Originally thought to exert its anti-proliferative effects through binding to the cell surface, CCN5—like CCN3—has recently been detected in the nucleus of cultured cells and in tissues. The role that nuclear CCN5 plays in the nucleus and the mechanism regulating nuclear targeting are the foci of our efforts. A CCN5 mRNA splice variant has been cloned from rat ovary. This mRNA splice variant lacks exon 3, which encodes rat CCN5 IGFBP domain (I), and due to a shift of the opening reading frame this splice variant produces a non-secreted truncated CCN5 containing only the VWC and TSP1 domains. While the full-length CCN5 is only expressed in growth arrested SMC, the splice variant is expressed in both growth arrested and exponentially growing cells. To further investigate the subcellular localization and potential function of CCN5 isoforms, we investigator constructed a series of plasmids expressing each domain of rat CCN5, individually and in combinations. Additionally, each construct was made in a secretable and non-secretable form, and tagged at both the N-terminal and C-terminal ends with FLAG and Myc epitopes, respectively. Using fluorescence microscopy, we simultaneously analyzed N-terminal FLAG tag and C-terminal Myc tag in transfected NIH 3T3, HEK293, C2C12, and COS7 cells. The non-secreted forms of the VWC domain (V) alone, TSP1 domain (T) alone, and IV, VT and IVT domain combinations are imported into nucleus, whereas the same constructs containing an N-terminal signal sequence are not detected in nucleus. Interestingly, the I domain alone and the IT domain combination are never observed in the nucleus, regardless of the presence or absence of a secretion signal. We also tested an IVT construct of rat CCN5 that was mutated at the only predicted nuclear localization sequence (NLS) in the V domain. The mutant IVT construct was still capable of importing into the nucleus in the cell lines tested above. Studies to determine how these isoforms are targeted to the nucleus and to assess their role in cell proliferation and motility are underway. To complement the domain construct approach, we have also developed several monoclonal antibodies directed against specific domains of CCN5. They will be used in conjunction with other CCN5

antibodies already available in our lab to analyze the expression pattern of CCN5 isoforms in smooth muscle cells and in a variety of cancers using tissue microarray. These studies should provide important insights into the mechanism of action of CCN5.

POLY(ADP-RIBOSE) POLYMERASE-1 (PARP-1) AND PARP-1 BINDING ELEMENT (PBE) ENHANCE MURINE CCN2 GENE TRANSCRIPTION IN PROXIMAL TUBULAR EPITHELIAL CELLS (PTEC)

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Since we had found that PTEC expressed profibrotic protein CCN2 in the fibrous kidney, we began to investigate the requirements for the epithelial cell-selective induction of CCN2. Transient transfection experiments with luciferase reporter minigenes bearing various fragments of the murine CCN2 promoter region and cultured PTEC revealed that the −455 to −434 bp region contained novel, positive cis-elements for CCN2 gene transcription. This 20 bp fragment bound to nuclear extracts from either basal or TGFb-1-activated PTEC, and yielded a shifted band in the DNA mobility shift assay. In addition, mutation in this 20 bp region lowered CCN2 promoter activities in basal as well as TGFb-1-activated PTEC (50% and 30%, respectively) while mutation in either Smad binding element (SBE) or basic control element-1 (BCE-1) in the CCN2 promoter lowered them only in TGFb-1-activated PTEC (30%). These results suggested that this 20 bp region enhances CCN2 gene transcription in basic and TGFb-1-activated PTEC, and this 20 bp region and SBE as well as BCE-1 are necessary for the full enhancement of CCN2 gene transcription in TGFb-1-activated PTEC. By protein mass fingerprint analysis, we identified PARP-1 as a trans-factor protein that binds to this 20 bp region. Our finding that knocking-down of PARP-1 mRNA by antisense oligoDNA transfection, but not treatment with a polyribosylation inhibitor, significantly decreased CCN2 gene transcription in cultured PTEC suggested that PARP-1 enhances CCN2 gene transcription likely via its physical presence. Therefore, we named this 20 bp cis-element as PBE. These machineries were also found to be active in vivo since knocking-down of PARP-1 mRNA in PTEC significantly reduced levels of CCN2 mRNA and blocked fibrogenesis in the kidney with ureter ligation. Thus, PARP-1 and PBE are of importance for full induction of CCN2 gene transcription in PTEC by TGFb-1, and likely to be targets for anti-fibrosis therapy since CCN2 is a main mediator of profibrotic effects of TGFb-1 in the fibrous kidney.

ROLES OF CCN2 IN SKELETAL GROWTH AND REGENERATION—REQUIREMENT FOR BOTH ENDOCHONDRAL AND INTRAMEMBRANOUS BONE FORMATION-

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Using mainly in vitro system, we have shown that CCN2, which is highly expressed in (pre)hypertrophic chondrocytes, plays an important role in endochondral ossification by acting on chondrocytes, osteoblasts and endothelial cells. To confirm these *in vitro* findings *in vivo*, we generated transgenic mice (tg) overexpressing the *ccn2* gene in cartilage under the control of the type II collagen (*Col2a1*) promoter. As a result, the body size of tg mice was enlarged owing to prolonged diaphyses of long bones, corresponding to the over-expression level of *ccn2* mRNA. Supporting these results, cultured chondrocytes isolated from tg mice expressed enhanced levels of *col2a1*, *col10a1,* and *aggrecan* mRNA and showed increased accumulation of proteoglycans. A histochemical analysis of tibia from 17.5E and P1 revealed that the deposition of proteoglycans and type II collagen increased in transgenic cartilage. Micromass cultures prepared from tg E11.5 limb bud cells showed accelerated cartilaginous nodule formation in comparison to wt limb bud cells, indicating stimulation of chondrogenesis by overexpressed CCN2. Furthermore, expression of vascular invasion factors such as *vegf* and *mmp9* mRNA was also enhanced. In addition, cell proliferation in the resting and proliferative zone of cartilage was increased, and enhanced apoptosis in the zone of cartilage-bone transition was observed. Moreover, bone mineral contents and thickness of cortical bone were increased in tg mice. These findings indicate that overexpression of CCN2 in cartilage accelerates endochondral ossification by promoting proliferation and initial to terminal differentiation of chondrocytes, resulting in prolonged long bones.

In contrast to endochondral ossification, little is known concerning the role of CCN2 during intramembranous bone formation. To investigate the role of CCN2 in intramembranous bone development, a comparative analysis of wild-type and *Ccn2* null mice was conducted. Multiple histochemical methods were employed to analyze the effects of CCN2 deletion *in vivo* and effects of CCN2 on the osteogenic response were evaluated with the isolated and cultured osteoblasts. As a result, we found a drastic reduction of the osteoblastic phenotype in *Ccn2* null mutants. Importantly, addition of exogenous CCN2 promoted every step of osteoblast differentiation and rescued the attenuated activities of the *Ccn2* null osteoblasts. These results suggest that CCN2 is also required for the normal intramembranous bone development.

In addition to normal skeletal development, administration of CCN2-gelatin hydrogel complex together with collagen scaffold to artificial bone defect of rat model revealed an important role of CCN2 in skeletal regeneration.

SKELETAL PHENOTYPE IN TRANSGENIC MICE OVER-EXPRESSING CTGF IN CELLS OF THE OSTEOBLAST LINEAGE

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CTGF has recently emerged as an important growth factor in osteogenesis, demonstrated by its ability to promote proliferation, matrix production and differentiation in cultures of osteoblasts. Since most of the data concerning the role of CTGF in osteogenesis has come from in vitro studies, in this study we generated transgenic mice in which CTGF is over-expressed under control of the truncated 3.6 kb collagen type 1 (pOBCol3.6) promoter (CTGF $^{pOBCol3.6}$ mice). This promoter was chosen because it is expressed early during osteoblast differentiation. The targeting vector used to generate transgenic mice also contained LacZ (to identify cells expressing the transgene) and an enhancer element to

boost CTGF expression. The presence of the transgene was determined by PCR of tail DNA using transgene specific primers. Six lines were established by mating founder mice with C57/Blk6 wild type (WT) mice. Multiple tissues were used to examine specificity of transgene expression using PCR with transgene specific primers, followed by confirmation of CTGF mRNA expression levels by Northern blot analysis. Transgene expression was highest in long bone and calvaria, with lower levels of expression in other type I collagen producing tissues (lung and skin). Two of the transgenic lines with different CTGF expression levels were used for analysis of the skeletal phenotype. Mice from one line survive, however, mice from the other line die within a few days after birth. Line one showed a 3-4 fold (moderate expression) increase and line two showed a >7-8 fold (high expression) increase in CTGF protein levels in bone when compared to age matched WT mice. Histological and morphometric examination of the distal femoral metaphysis from TG mice with moderate over-expression of CTGF exhibited significant increases in trabecular bone volume associated with increased osteoid thickness and osteoblast activity/numbers compared to WT mice. Increased thickness of the periosteum with increased numbers of osteoprogenitor cells was also observed in TG compared to WT bone. Primary cultures of osteoblasts derived from these TG mice also exhibited enhanced differentiation (ALP staining and mineralization) compared to WT cultures. Surprisingly, examination of bones from transgenic mice over-expressing CTGF at very high levels demonstrated an increase in osteoclast number and size. These data suggest that the precise effects of CTGF on bone cell differentiation and function depend on the magnitude of CTGF over-expression. Moderate levels of CTGF have a direct effect on osteoblasts to promote bone formation, while high levels favor the formation of osteoclasts, perhaps indirectly through a RANK-L dependent mechanism.

CCN1 AND CCN2 ARE ESSENTIAL FOR CHONDROGENESIS

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CCN1 (Cyr61) and CCN2 (CTGF) are essential for many aspects of development. CCN1 is a potent angiogenic factor and its global deletion results in early embryonic lethality due to defects in angiogenesis and vasculogenesis. CCN1 null mice display defects in

Integrin-mediated cellular adhesion and in the matrix production that induces this adhesion. A global deletion of CCN2 results in perinatal lethality due to improper chondrogenesis. *Ccn2* null defects are attributed to impaired hypertrophic chondrocyte proliferation, extracellular matrix production and vascularization. Both CCN1 and CCN2 regulate specific aspects of angiogenesis and bone formation in vivo. However, the role of CCN1 in chondrogenesis in vivo is unknown, nor is it established whether CCN1 and CCN2 have overlapping functions in skeletal development. We have generated mice lacking both CCN1 and CCN2 specifically in cartilage to address these issues. Cartilage specific loss of CCN1 leads to perinatal lethality, but the skeletons of $Ccn1^{CKO}$ (CKO = cartilage-specific knockout) mice exhibit only minor alterations. However, *Ccn1/Ccn2* double mutants exhibit perinatal lethality and a skeletal phenotype that is more severe than that of *Ccn1* and *Ccn2* null mice. *Ccn1/Ccn2* double mutants display global chondrodysplasia. The growth plates of double mutants exhibit an expansion of the hypertrophic zone and a decrease in trabecular and perichondrial bone formation, which is indicative of defects in chondrocyte differentiation, clearance and vascular invasion. Combined, this evidence demonstrates that CCN2 is required for chondrogenesis and that there is functional overlap between CCN1and CCN2 in endochondral bone formation.

CCN3 AND NOTCH/BMP SIGNALS

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CCN3 inhibitory effect on osteogenesis has been reported from many groups and the recent reports using transgenic mice (gain/loss of function) strongly confirmed it from the aspect of body formation. CCN3 effect is believed to relate to several different signal cascades such as BMP, Wnt or Notch, but still the priority of the used signals is unclear. The group of Canalis concluded the BMP signal has the priority among these signals, but our results demonstrated the importance of Notch signal. To investigate these molecular talks, we constructed deletion forms of CCN3 and transfected to Kusa-A1, a mouse osteogenic mesenchymal stem cell line. A CT domain deleted form (delCT) has shown a decreased osteogenic activity, but did not change the neurogenic activity. Previously, we confirmed that CT domain is responsible for Notch interaction, but not for CCN3 dimerization. Therefore, it was possible to hypothesize that the reduction of osteogenesis might be due to the deceased Notch activity. But we evaluated downstreams of Notch signal and verified that Hey1 expression was attenuated by delCT transfection. We also demonstrated that delCT still possessed the BMP binding activity, indicating its masking effect. In fact, delCT showed a decreased phosphorylation of SMAD proteins when transfected. CCN3 expression has been investigated in osteoblast cell lines in other groups, but we employed a bone marrow-derived mesenchymal stem cell line, which naturally expresses CCN3. We conclude that inhibitory effect of CCN3 against osteogenesis is communicated by both Notch and BMP signals.

FAK/SRC SUPPRESSES EARLY CHONDROGENESIS: CENTRAL ROLE OF CCN2

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Adhesive signaling plays a key role in cellular differentiation, including in chondrogenesis. Herein, we probe the contribution to early chondrogenesis of two key modulators of adhesion, namely FAK/src and CCN2 (Connective tissue growth factor, CTGF). We use the micromass model of chondrogenesis to show that FAK/src signaling, which mediates cell/matrix attachment, suppresses early chondrogenesis including the induction of *Ccn2, Agc* and *Sox6*. The FAK/src inhibitor PP2 elevates *Ccn2, Agc* and *Sox6* expression in wild-type mesenchymal cells in micromass culture, but not in cells lacking CCN2. Our results suggest a critical feature permitting chondrogenic differentiation is a reduction in FAK/src signaling, and that CCN2 operates downstream of this loss to promote chondrogenesis.

CTGF/CCN-2 IS PRODUCED BY NON-CHONDRODYSTROPHIC CANINE INTERVERTEBRAL DISC-DERIVED NOTOCHORDAL CELLS AND UPREGULATE NUCLEUS PULPOSUS AGGRECAN GENE EXPRESSION

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Non-chondrodystrophic (NCD) dogs maintain large populations of notochord cells within their intervertebral discs for many years and are not known to develop degenerative disc disease until much later in life. Chondrodystrophic breeds develop disc disease much earlier and they have a paucity of such notochord cells. We have previously reported upregulation of aggrecan, versican and hyaluronic acid synthase-2 genes as a consequence of treatment of nucleus pulposus cells treated with notochordal cell conditioned medium. We were the first to demonstrate that notochordal cells secrete connective tissue growth factor (CTGF/CCN-2). CCN-2 has been shown to stimulate healing of defects in articular cartilage and has been hypothesized to be of potential utility in the treatment of damage to articular cartilage. Since we have identified the presence of CCN-2 in NCCM, we chose to examine aggrecan gene expression as a function of culturing bovine intervertebral disc-derived chondrocytes with known doses of recombinant human CTGF as well as NCCM and minimal media devoid of anabolic substances (DMEM). Further, we have demonstrated the presence of CTGF in notochordal cell lysates using Western blotting methods.

Here we demonstrate that canine notochord cells (obtained from NCD dogs) were cultured within alginate beads in serum-deficient conditions (DMEM) to produce notochord cell conditioned medium (NCCM). Bovine disc-derived chondrocytes were obtained and cultured for 3 days in totally serum-free medium and then cultured for 24 h with DMEM, NCCM and DMEM + doses of 50, 100, and 200 ng/mL as well as NCCM + 200 ng/mL of recombinant human CTGF (rCTGF or rCCN-2). After 24 h, total RNA was extracted (Trizol) from the chondrocytes, the RNA was quantified at $OD_{260/280}$ and then 1 μg total RNA was reverse transcribed. The resulting cDNA obtained was subjected to semi-quantitative RT-PCR using aggrecan specific primers.

We demonstrated that aggrecan gene expression was modestly produced in the chondrocytes treated with DMEM only however in a dose-dependent relationship aggrecan gene expression was robustly increased by rCTGF and NCCM as compared to DMEM. NCCM induced chondrocyte aggrecan gene expression at a similar level to between 100 and 200 ng/mL rCTGF. NCCM +200 ng/mL CTGF resulted in similar upregulation of aggrecan as 200 ng/mL alone.

CCN2 IS REQUIRED FOR VASCULAR REMODELING IN VIVO

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Numerous *in vitro* studies have demonstrated that CCN2 regulates angiogenesis. However, both pro-and anti-angiogenic effects have been reported. Hence, we examined *Ctgf-/-* mice as well as mice lacking *Ccn2* specifically in endothelial cells for angiogenic phenotypes. This analysis revealed that the vascular system develops normally initially in *Ccn2-/-* mice, but vascular remodeling is defective. Analysis of endothelial-specific *Ccn2* knockouts, generated using VE-CAD-Cre, showed that the vascular defects are due to loss of *Ccn2* in endothelial cells. Moreover, the endothelial-specific knockout mice die perinatally, demonstrating that *Ccn2* is required for vasculogenesis *in vivo*. Histological analysis reveals that the vascular defect is caused by insufficient pericyte recruitment and stabilization of endothelial-pericyte interactions. Furthermore, the defect in pericyte recruitment is related to a defect in endothelial cell shape; in mutants, endothelial cells are tortuous and exhibit numerous abluminal protrusions. Electron microscopy confirmed this hypothesis, and revealed that endothelial basement membranes are abnormal in *Ccn2-/-* mice. Analysis of specific ECM components revealed that the major components of basement membranes are expressed at normal levels in *Ccn2* mutants, but that their assembly into a cohesive basement membrane is defective. These results reveal an essential for CCN2 in basement membrane assembly, and raise the possibility that CCN2 is an essential regulator of epithelial-mesenchymal interactions and maintenance of stem cell niches that involve basement membranes.

KLF15 REGULATES THE CARDIAC RESPONSE TO STRESS

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Cardiac hypertrophy and fibrosis is a common response to injury and hemodynamic stress and an important harbinger of heart failure and death. Herein, we identify the Kruppel-like factor 15 (KLF15) as a novel regulator of the heart's response to stress. KLF15 is expressed in both cardiomyocytes and fibroblasts. KLF15 expression is reduced by a broad spectrum of pro-hypertrophic agents in cardiomyocytes (phenylephrine, Tgfb1, Ang II) and pro-fibrotic agents (Tgfb1, Ang II). Consistent with this observation, myocardial expression of KLF15 is reduced in rodent models of hypertrophy and in biopsy samples from patients with pressure-overload induced by chronic valvular aortic stenosis.

Studies in cardiomyocytes indicate that sustained expression of KLF15 in neonatal rat ventricular cardiomyocytes (NRVMs) inhibits cell size, protein synthesis and hypertrophic gene expression. Mechanistically, a combination of promoter analyses (ANF and BNP) and gel-shift studies suggest that KLF15 can inhibit GATA4 and MEF2 function. Studies in neonatal rat ventricular fibroblasts (NRVFs) indicate that sustained expression of KLF15 inhibited basal and TGFb1-induced CTGF expression—a key regulator of tissue fibrosis. To determine the molecular basis for KLF15's ability to inhibit CTGF expression, promoter analyses were undertaken. KLF15 inhibited basal and TGFb1-mediated induction of the CTGF promoter activities. Previous studies indicate that TGFb1-mediated induction of CTGF occurs via Smad3 as well as via the co-activator P/CAF. Using a combination of ChIP and electrophoretic mobility shift assays, we show that that while KLF15 has no significant effect on Smad3 binding to CTGF promoter, it strongly inhibits recruitment of P/CAF to CTGF promoter. Consistent with this observation, KLF15 mediated inhibition of CTGF was rescued by overexpression of P/CAF.

To elucidate the role of KLF15 in cardiac biology in vivo, KLF15-null mice were generated. KLF15-null mice are viable but, in response to pressure overload, develop an eccentric form of cardiac hypertrophy characterized by increased heart weight, exaggerated expression of hypertrophic genes (ANF, BNP), left ventricular cavity dilatation with increased myocyte size and reduced left ventricular systolic function. Furthermore, hearts from KLF15 (-/-) mice subjected to aortic banding exhibited increased CTGF levels. Trichrome staining also suggested elevated deposition of collagen protein in KLF15 (-/-) heart in response to mechanical stress. These data identify KLF15 as part of a heretofore unrecognized pathway regulating the cardiac response to hemodynamic stress.

THE ROLE OF *CTGF* IN PAEDIATRIC ACUTE LYMPHOBLASTIC LEUKAEMIA

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Acute lymphoblastic leukaemia (ALL) is the most common form of cancer in children. It is a heterogenous disease, initiated by a range of genetic events that give rise to multiple clinical subtypes with varying prognoses. Although survival rates are approaching 80%, a significant number of patients continue to relapse and the outlook for these is dismal. In order to improve outcome novel therapeutic strategies are required. Leukaemias arise in the haemopoietic cells of the bone marrow and this microenvironment plays a major role in the disease. Using microarray technology we compared the gene expression profile of ALL to normal $CD34^+$ cells separated from bone marrow, and we identified a set of highly differentially expressed genes. Many of the top-ranked genes are known to mediate cell–cell interactions, including *ECM1, EFNB2, BMP2* and *CTGF*. Four independent studies on B-lineage ALL in paediatric and adult patients showed that 75% of specimens consistently expressed *CTGF* at very high levels. In our paediatric patient specimens the gene was expressed over a wide range, from 2.3–to 380-fold by array measurement. Our current studies focus on the mechanisms leading to high *CTGF* expression. In order to gain insight into the role of *CTGF* in leukaemia we studied ALL cell lines established from paediatric patients and demonstrated secreted CTGF of 30 kDa and 38 kDa, however the proliferation of ALL cells was not enhanced in the presence of recombinant human (rh) CTGF. In contrast, bone marrow stromal cells showed a dose-dependent proliferative response to rhCTGF, suggesting that a paracrine mechanism may be involved. We examined the gene expression of bone marrow stromal cells incubated with rhCTGF and identified prominent signatures implicated in the regulation of cell–cell interactions and proliferation. In order to test strong adhesion (against gravity) we designed a closed culture system and monitored adhesion by flow cytometry under various experimental conditions. The presence of rhCTGF mediated enhanced adhesion of ALL cells. Our current studies focus on the functional role of the signalling molecules implicated by our studies, including resistance to drug therapy. Activation and secretion of CTGF play a prominent role in ALL, leading to modified interactions with the microenvironment, and these processes are thought to promote the growth of pre-leukaemic cells. Improved understanding of the CTGF-mediated changes in pre-malignant and malignant cells in the bone marrow microenvironment is expected to lead to better therapeutic strategies for patients with ALL.

EXPRESSION OF CCN PROTEINS IN NORMAL HUMAN SKIN *IN VIVO* AND AFTER WOUND HEALING

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CCN proteins have emerged as important and ubiquitous modulators of development and adult organ function. To date, CCN protein distribution and function in adult human skin has not been carefully examined. Human skin is comprised of multiple cell types of ectodermal, mesodermal, or neural-crest origin. In this study, we examined localization and expression of the six CCN proteins in normal adult human skin and during early stages of wound healing *in vivo*, in both dermal and epidermal compartments of the skin. Transcript and protein expression were studied by laser-capture microdissection-coupled real-time RT-PCR and immunohistochemistry, respectively. First, we studied normal human skin to determine localization and expression levels of CCN proteins. Our results demonstrate that among the six CCN proteins, CCN2, CCN3, and CCN5 were most highly expressed in the epidermis. CCN3 and CCN5 proteins were most prominent in epidermal keratinocytes, whereas CCN2 was primarily expressed by melanocytes. Differential expression within epidermal layers suggests that CCN3 and CCN5 are linked with keratinocyte differentiation. CCN2 and CCN5 mRNA were the most highly expressed in the dermis. CCN3 mRNA was ~3 fold lower than CCN2/5, CCN1/4/6 transcripts were expressed at similar levels, which were approximately 200-fold lower than those of CCN2/5. Next, we studied alterations of CCN mRNA and protein expression during reepithelialization and dermal remodeling, following skin wounding. Wounding was accomplished by thermal ablation of the epidermis of normal forearm skin, by CO_2 laser. Our data show that, in addition to being spatially regulated, CCN proteins are temporally and specifically regulated during different phases (inflammation, proliferation, and remodeling) of wound healing. CCN1 and CCN4 expression gradually increased, while CCN5 decreased, in the dermis during the first week of wound healing. CCN1 was localized to blood vessels. CCN2 increased in the dermis during later stages of remodeling, in association with deposition of new extracellular matrix. In contrast, CCN3 expression was substantially decreased in epidermis and dermis during the phase of intense cellular proliferation. CCN6, expressed at low levels in adult human skin, was not altered during wound healing. Taken together, these data demonstrate cell type specific expression of CCN proteins in human skin, and stage-dependent regulation during wound healing. These data suggest that CNN family members exert distinct functional roles in the epidermis and dermis of human skin in vivo.

CONNECTIVE TISSUE GROWTH FACTOR PROMOTER ACTIVITY IN NORMAL AND WOUNDED SKIN

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In skin, connective tissue growth factor (CTGF/CCN2) is induced during tissue repair. However, the exact cell types in which CCN2 is expressed in normal and wounded skin remains controversial. In this report, we use transgenic knock-in mice in which the Pacific Jellyfish *Aequorea victoria* gene enhanced green fluorescent protein (E-GFP) is inserted between the endogenous CCN2 promoter and gene. Unwounded (day 0) and wounded (days 3 and 7) skin was examined for GFP (to detect cells in which the CCN2 promoter was active), a—smooth muscle actin (a-SMA) (to detect myofibroblasts), and NG2 (to detect pericytes) expression. In unwounded mice, CCN2 expression was absent in epidermis and was present in few cells in the dermis. Upon wounding, CTGF was induced in the dermis. Double-immunolabeling revealed that CCN2-expressing cells also expressed a-SMA, indicating CCN2 was expressed in myofibroblasts. A subset (~30%) of myofibroblasts was also NG2-positive, indicating that pericytes significantly contributed to the number of myofibroblasts in the wound. Pericytes expressed CCN2. Collectively, these results indicate that CCN2 expression in the skin correlates with myofibroblast induction, and that CCN2-expressing pericytes are significant contributors to myofibroblast activity during cutaneous tissue repair.

ULTRAVIOLET IRRADIATION INDUCES CYR61/CCN1, A NOVEL MEDIATOR OF COLLAGEN HOMEOSTASIS, VIA ACTIVATION OF TRANSCRIPTION FACTOR AP-1 IN HUMAN SKIN FIBROBLASTS

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Ultraviolet (UV) radiation from the sun damages skin connective tissue dermis and causes premature skin aging (photoaging). This photodamaged skin is largely caused by imbalance of collagen homeostasis, characterized by elevated production of collagen-degrading matrix metalloproteinases (MMP) and reduced production of new collagen. This aberrant dermal collagen homeostasis is mediated in part by CCN family member, cysteine-rich protein 61 (CYR61/CCN1). CYR61 functions as a novel mediator of collagen homeostasis by inhibiting type I collagen production, the major structural protein in human skin, and promoting its degradation. CYR61 is significantly elevated in the dermis of photoaged human skin, acutely UV-irradiated human skin in vivo, and UV-irradiated human skin fibroblasts. Inhibition of UV-induced CYR61 by CYR61 siRNA significantly attenuated UV-induced inhibition of type I procollagen and up-regulation of matrix metalloproteinase-1 (MMP-1), indicating CYR61 functions as a novel mediator for UV-induced aberrant collagen homeostasis. UV irradiation significantly activates CYR61 promoter without changing the stability of CYR61 mRNA and protein, indicating the primary mechanism of CYR61 induction by UV irradiation is transcriptional. Analysis of CYR61 promoter revealed that CYR61 proximal promoter contains functional AP-1 binding site. Analysis of proteins bound to the AP-1 site revealed that UV irradiation increased binding of AP-1 family members, c-Jun and c-Fos. Deletion or mutation of AP-1 binding site in the CYR61 promoter near completely abolished UV activation of CYR61 promoter. Furthermore, functional blocking of c-Jun or knockdown c-Jun substantially reduced UV-induced activation of CYR61 promoter and CYR61 expression. These data demonstrate that CYR61 is transcriptionally regulated by UV irradiation via activation of transcription factor AP-1, and functions as a novel mediator for solar UV radiation-induced dermal connective tissue damage.

THE INTERACTION OF β-CATENIN WITH EXTRA-CELLULAR MATRIX COMPONENTS IN DERMAL FIBROBLASTS DURING WOUND HEALING

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During cutaneous wound healing, dermal fibroblasts synthesize new extracellular matrix (ECM) to repair the skin. CCN family members (most notably CCN2, also called connective tissue growth factor) play a role in this process. β-catenin binds and stimulates tcf transcription factors, and it is activated in wound fibroblasts. However, the factors that regulate β-catenin during healing, and its transcriptional targets are not well understood. One possibility is the involvement of the ECM as both a target and a regulator of β-catenin activity. We are investigating the existence of a reciprocal regulatory loop between β-catenin and ECM during healing. The expression pattern of several ECM components, which include β-catenin transcriptional targets, was verified using real time PCR analysis in mouse full thickness wounds. To investigate the effect of ECM on β-catenin, primary dermal fibroblasts from tcf-reporter mice were cultured on collagen I, IV, fibronectin, and matrigel. We found an increase in activated β-catenin protein and in tcf-dependent transcription compared to plastic. Inhibition of glycogen synthase kinase 3β, which mediates the destruction of β-catenin, was demonstrated in fibroblasts grown on ECM. The common ECM-integrin binding motif, RGDS, which activates integrins that also interact with CCN2, caused an elevation in active β-catenin protein. This was examined *in vivo* using fibronectin deficient mice, in which a higher level of β-catenin was observed during wound repair. To identify ECM-associated targets of β-catenin during wound healing, we are using expression array analysis to compare gene expression in mice with conditionally regulated β-catenin versus 'wild-type' levels. Our results to date suggest that numerous ECM and adhesion molecules are differentially expressed in the wounds of mice with conditionally stabilized β-catenin compared to 'normal' wounds. Thus, ECM upregulates β-catenin protein and transcriptional activity and β-catenin modulates the expression of ECM components, suggesting that the ECM acts in a feedback loop during wound repair to regulate β-catenin. Elucidating the mechanism of interaction between β-catenin and the ECM during healing may generate new therapies to treat wound healing disorders.

THE ROLE OF THE MATRICELLULAR PROTEIN DEL1 IN BONE FRACTURE HEALING

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DEL1 is a 52 kDa secreted, extracellular matrix-associated protein whose structure is notable for three EGF-like repeats, two discoidin-like domains, and an RGD motif. The protein has been shown to bind integrins avb3 and avb5, and to induce angiogenesis in *in vitro* assays. During development, *Del1* mRNA is expressed in developing vascular structures. We have found very prominent *Del1* expression in cartilaginous tissues. Expression can be seen in hypertrophic cartilage as well as mature cartilage. These data about DEL1's biochemistry and developmental pattern of expression are strikingly similar to published data on CYR61 (CCN1) and CTGF (CCN2) raising questions about their *in vivo* biological role.

Transgenic DEL1 null mutant mice appear normal, are of equal size to wild type littermates, and are fertile. Litters from heterozygote parents show expected Mendelian ratios suggesting there is no effect on embryonic viability. Skeletal dissections and radiographs did not reveal any obvious abnormalities in null mutant mice. Using a tibial fracture model, we detected decreased bony callus formed in the null mutant mice compared to wild type littermates using radiographs and microCT.

Using purified DEL1, we found it to be a potent inhibitor of apoptosis induced through either intrinsic or extrinsic pathways in primary chondrocytes with no effect on chondrocyte proliferation. Inhibition of apoptosis was through integrin binding and required the RGD motif. Using antibodies that blocked binding to specific integrins, we found that inhibition of apoptosis requires binding to integrin avb5, but not avb3. The anti-apoptotic effect of Del1 was not blocked by soluble proteoglycans including heparin, chondroitin and dermatan suggesting that proteoglycans are not required as co-factors in binding.

Bone forms during development through endochondral ossification in the majority of the skeleton, except in the skull plates where membranous ossification takes place. During endochondral ossification, bone forms through an intermediate of hypertrophic cartilage whereas there is no cartilaginous intermediate in membranous bone formation. In our fracture model, fracture healing occurs through endochondral ossification. We propose that Del1 acts to prevent premature apoptosis of the hypertrophic cartilage in the fracture callus leading to more robust bone formation. These findings are of interest in comparisons with CYR61 and CTGF null mutants phenotypes, and suggest that despite similar receptors and *in vitro* activity, additional factors are at play in determining the *in vivo* biological role.

CONNECTIVE TISSUE GROWTH FACTOR (CTGF/CCN2) AS A REGULATOR OF β1 INTEGRIN-MEDIATED CELL ADHESION AND SURVIVAL DURING LACTOGENIC DIFFERENTIATION OF MOUSE MAMMARY EPITHELIAL CELLS

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The degree of differentiation of mammary epithelial cells is dependent on their response to basement membrane and stromal protein-induced signals. Our previous work determined that connective tissue growth factor (CTGF/CCN2), a known stromal mediator, was highly up-regulated during lactogenic differentiation of mouse mammary epithelial cells. CTGF/CCN2 is transcriptionally regulated by dexamethasone, and is required for lactogenic differentiation of the HC11 mouse mammary epithelial cell line. Using a CTGF/CCN2 Tet-off system in the HC11 cell background, elevated CTGF/CCN2 expression enhanced multiple markers of lactogenic differentiation including β-casein transcription, mammosphere formation, and stabilization of Stat5 phosphorylation. Infection of primary mouse mammary epithelial cells with replication defective adenovirus encoding CTGF/CCN2 induced similar responses. Elevated CTGF/CCN2 expression decreased the requirement for extracellular matrix components including collagen I, fibronectin, and laminin for initiation of β-casein transcription. Because β1 integrin signaling is required for lactogenic differentiation and the transcription of β-casein, the contribution of CTGF/CCN2 expression to the formation and stability of the β-casein promoter transcription complex has been examined by chromatin immune-precipitation (ChIP). The effect of CTGF/CCN2 on both Stat5 and the SWI-family protein, Brg1, binding and function at the b-casein promoter will be reported.

CTGF/CCN2 expression both enhanced cell growth and survival and prevented apoptosis of mammary epithelial cells. HC11 cells expressing CTGF/CCN2 exhibited an increase in the level of β1 integrin and downstream survival signaling mediators including phospho-FAK, phospho-Akt, and cyclin D1. Elevated CTGF/CCN2 levels increased the formation of focal adhesion complexes, as viewed by immunofluorescence, and increased expression levels of focal adhesion-related adaptor, structural, and signaling proteins including parvin, p130cas, paxillin, Src, vinculin, and integrin-linked kinase. These results demonstrate that the mechanism by which CTGF/CCN2 contributes to lactogenic differentiation is via activation of β1 integrin-mediated adhesion complexes and integrin-dependent signaling pathways.

CCN2/CTGF–CARDIOPROTECTIVE FACTOR IN MYOCARDIAL ISCHEMIA/REPERFUSION INJURY AND HEART FAILURE

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Background: In physiologic postnatal life expression of myocardial CTGF/CCN2 is repressed. However, myocardial CTGF is dramatically induced in heart failure. Yet, the physiologic and pathophysiologic roles of myocardial CTGF remain unresolved.
Methods and Results: To elucidate the actions of myocardial CTGF and its putative role in heart failure, transgenic mice with cardiac-restricted (α-MHC promoter) overexpression of CTGF were generated. Transgenic CTGF (Tg-CTGF) mice had slightly smaller cardiac mass than that non-transgenic littermate controls (NLC) (heart weight/body weight ratio; 4.5±0.1 vs. 5.2±0.1 mg/g in Tg-CTGF vs. NLC, $p<0.05$). Consistently, echocardiography revealed slightly smaller left ventricular (LV) dimensions in Tg-CTGF vs. NLC mice. Simultaneous LV pressure-volume analysis in vivo did not disclose significant alterations of contractility and cardiac output, or evidence of left ventricular dysfunction in Tg-CTGF mice. Analysis of myocardial gene expression by DNA microarray revealed a gene expression signature of Tg-CTGF mice consistent with inhibition of myocardial growth, and activation of genes that confers cardioprotection towards ischemia/reperfusion injury, as well as activation of genes that encode extracellular matrix proteins. In order to test the functional significance of these gene expression signatures in cardiac pathophysiology, hearts from Tg-CTGF and NLC mice were subjected Langendorff perfusion *ex vivo* and 40 min of global ischemia succeeded by 60 min of reperfusion. Infarct size was markedly diminished in Tg-CTGF vs. NLC hearts and recovery of LV developed pressure was enhanced. Consistently, recombinant hCTGF included in the perfusion buffer prior to ischemia also conferred cardioprotection and diminished infarct size. Tg-CTGF mice subjected to pressure-overload by abdominal aortic banding displayed diminished hypertrophy and preserved LV pressure-volume relations after 12 weeks compared with NLC mice subjected to similar aortic constriction.
Conclusion: This study discloses the novel findings that CTGF exerts antihypertrophic actions in the heart, delays onset of heart failure following aortic constriction, and confers cardioprotection by preemptive preconditioning due activation of salvage kinase signaling pathways and reprogramming of gene expression.

HIGH GLUCOSE AND FREE FATTY ACID ADVERSE EFFECTS ON CARDIAC MYOCYTES ARE MEDIATED BY CCN2/CONNECTIVE TISSUE GROWTH FACTOR (CTGF) THROUGH TrkA

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Diabetic cardiomyopathy is characterised by interstitial fibrosis, cardiomyocyte hypertrophy and apoptosis. CCN2, also known as CTGF, is implicated in the fibrosis however whether it contributes to the cardiomyocyte changes, or to documented adverse effects of high glucose and lipids on these cells, remains unknown.

H9C2 cardiomyocytes were treated with recombinant human (rh) CTGF, high glucose or the saturated fatty acid, palmitate. Each reagent induced cell hypertrophy, as indicated by the ratio of total protein /cell number and the gene expression of cardiac hypertrophy marker genes ANP and α-skeletal actin, by qRT-PCR. Data shown are at 24 h and similar effects occurred at 48 h. Each treatment also caused apoptosis, as measured by increased caspase3/7 activity and a lower viable cell number. An osmolality control had no effect on these end-points.

Treatments	Hypertrophy Markers (% of control)			Apoptosis Markers (% of control)	
	Total protein/cell No.	ANP mRNA	α-sk actin mRNA	Caspase 3/7 Activity	Viable cell number
rhCTGF (500 ng/mL)	183±22*	145±16*	129±10*	179±12*	79±8*
Glucose (30 mM)	148±24*	340±43*	201±27*	228±5.0*	79±2*
Palmitate (50 μM)	165±29*	161±23*	155±18*	192±19*	68±5*
Palmitate (100 μM)	397±7.0*	143±24	189±39*	262±49*	53±5*
Glucose (30 mM) + CTGF SiRNA	114±16^{+}	ND	ND	156.3±17^{+}	88±8^{+}
Palmitate (50 μM) + CTGF SiRNA	107±1.8^{+}	ND	ND	123.5±35^{+}	98±7^{+}

Mean±SD; *P<0.05 compared with respective untreated control, ^{+}P< 0.05 compared with respective treatment+scramble SiRNA negative control by ANOVA; ND–not done. Further studies showed that CTGF mRNA was induced at 16 h by high glucose (30 mM) and palmitate (50 μM) to 162% and 300% of control, respectively (P<0.05). SiRNA (5 nM) against CTGF lowered steady state CTGF mRNA levels to 81% of control; it completely blocked the high glucose and palmitate induction of hypertrophy, and partially inhibited the up-regulated caspase 3/7 activity (Table). In contrast, a scrambled RNA control sequence had no effect on these parameters. In addition, these CTGF effects were through the trkA receptor with tyrosine kinase activity, which has previously been implicated in CTGF signalling: trkA was phosphorylated by CTGF in these cells by Western analysis, and a specific trkA blocker (100 nM k252a, Merck) abrogated CTGF-induced trkA phosporylation and CTGF effects on hypertrophy and apoptosis. Pre-incubation of cells with the cardioprotective incretin, glucagon-like peptide-1 (GLP-1) prevented the CCN2, high glucose and palmitate induced increase in caspase and the reduction in viable cell number. Collectively, this data implicates autocrine CTGF as a mediator in adverse effects of high glucose and fatty acids on cardiomyocytes, through trkA mediated mechanisms. *Supported by NHMRC Australia.*

BALANCING CCN-2 (CTGF) AND BMP(S) IN COMPLICATIONS OF DIABETES MELLITUS

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Recently, CCN-2 (connective tissue growth factor; CTGF) and bone morphogenetic proteins (BMPs) have emerged as key players in diabetic nephropathy. In experimental diabetic nephropathy, the expression of BMP-7 is decreased and BMP antagonists are increased, while BMP-7 treatment improves outcome.

On the other hand, CCN-2 is strongly upregulated in experimental and human diabetic nephropathy. In patients with diabetic nephropathy, both urinary CCN-2 excretion and plasma CCN-2 are increased and correlate with clinical markers of renal disease. The association of elevated CCN-2 levels with diabetic nephropathy was found to be at least as strong as that of the established risk factors hypertension and hyperglycemia. Baseline plasma CCN-2 was an independent predictor of end-stage renal disease and mortality in patients with diabetic nephropathy. Furthermore, in diabetic patients with nephrotic range albuminuria, plasma CCN-2 was the only parameter predicting end-stage renal disease. These observations suggest that CCN-2 in urine and plasma might find clinical application as a biomarker.

The involvement of CCN-2 in the pathogenesis of experimental diabetic nephropathy and its complications has been demonstrated by modulation of CCN-2 levels by genetic deletion and overexpression, and by treatment with CCN-2 antisense oligodeoxynucleotides or CCN-2 neutralizing antibody. These modulations significantly affected the magnitude of structural and functional changes, including matrix metalloproteinase activity, extracellular matrix accumulation, glomerular basement membrane thickening, albuminuria, serum creatinine, as well as cardiovascular function. Interestingly, CCN-2 was found to inhibit the renal signaling activity and target gene expression of BMP-7, both in diabetic mice and in cultured renal cells. Also in human diabetic nephropathy BMP signalling activity was diminished, together with reduction of podocyte markers. These changes were associated with overexpression of CCN-2 but not SOSTDC1.

In conclusion, CCN-2/CTGF emerges as a critical determinant of diabetic nephropathy and cardiovascular disease. At least in the kidney, it's role appears to involve inhibition of BMP-signaling activity. Better understanding of the interplay between CCN-2 and BMPs might guide development of novel biomarkers and therapeutic strategies, but also investigation of the impact of CCN-2 on other pathways will be important.

CCN2/CTGF IS TRANSACTIVATED THROUGH ITS ENHANCER ELEMENT BY SOX9 IN FIBROBLASTS: POSSIBLE ROLES IN FIBROSIS

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Accumulation of extracellular matrix proteins is one of the characteristic pathological changes in sclerotic fibroblasts. CCN2/CTGF which is expressed predominantly in prehypertrophic chondrocytes in the physiological state, but also in many types of fibroblasts under pathological conditions, is involved in the pathogenesis of sclerosis through strong induction of synthesis and accumulation of extracellular matrix components. Sox9, a transcriptional regulator for cartilage-specific extracellular matrix components, is essential for the chondrocytic cell fate and also strongly expressed in prehypertrophic chondrocytes. Here we propose that 1) Sox9 may regulate *ccn2/ctgf* expression in chondrocytes, and 2) that there may be a relationship between overexpression of CCN2/CTGF and enhanced Sox9 expression in fibroblasts and the pathogenesis of sclerosis.

In this report, we identified Sox9-enhancer regions in the *ccn2/ctgf* promoter by deletion analysis of *ccn2/ctgf* promoter-reporter constructs; mutations within the enhancer region abrogated promoter activitation by Sox9. Sox9 bound to the enhancer region of *ccn2/ctgf in vitro* as shown by gel shift assays. Chromatin immunoprecipitation analysis using Sox9 antibodies effectively precipitated the *ccn2/ctgf* enhancer, confirming Sox9-binding to the *ccn2/ctgf* enhancer *in vivo*. Conversely, embryonic fibroblasts from skin-specifically *ccn2/ctgf*-overexpressing mice showed enhanced expression of Sox9 and aggrecan mRNA in comparison to wildtype fibroblasts, and adult

skin in the transgenic mice showed expression of Sox9 and aggrecan proteins. Similarly, cartilage-specifically *ccn2/ctgf*-overexpressing mice showed enhanced expression of Sox9 and aggrecan mRNA in cartilage. These findings indicate a stimulatory feed back loop in which overexpression of CCN2/CTGF stimulates expression of Sox9, and the induced Sox9 further promotes CCN2/CTGF expression, resulting in accelerated CCN2/CTGF production and aggrecan accumulation. This may be the cue to the pathogenesis of experimental fibrosis.

IN VIVO EFFECTS OF CCN5 OVEREXPRESSION IN FIBROIDS

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Uterine leiomyoma (fibroids) are smooth muscle tumors that form in the myometrial layer of the uterus. They are the most common of all tumors in women and in the United States result in over 200,000 total abdominal hysterectomies in symptomatic patients every year. Clinically symptomatic fibroids are present in 15–20% of the general female population, and the prevalence rises to >60% in women of African decent. Currently, the only treatment proven to prevent recurrence of the disease is the relatively invasive procedure of total abdominal hysterectomy. The development of less drastic therapeutic alternatives requires a detailed understanding of the cellular and molecular mechanisms regulating the proliferation of both fibroid and normal SMC. Earlier work in our laboratory has shown that primary cultures of neoplastic SMC cells isolated from fibroids display no endogenous production of CCN5 protein, while SMC derived from the myometrium of the same patients display normal levels of CCN5. In addition, forced expression of CCN5 utilizing an adenoviral system inhibits the proliferation and motility of cultured fibroid cells. When CCN5 overexpression is attempted in an *in vivo* model similar results are found. Adenoviral overexpression of CCN5 in the rat leiomyoma cell line ELT-3 inhibits the *in vivo* formation of tumors by these cells and decreases tumor mass by 72%. Also of great interest to the field of fibroid research is the recent success our lab has had in the development of an *in vivo* leiomyoma model that utilizes human fibroid cells. Currently the field lacks a human-based *in vivo* system to study the effects of potential therapeutics such as CCN5. Using freshly isolated human fibroid tissue organoids suspended in a matrigel/collagen I mixture, a subcutaneous injection of the organoid suspension, followed by whole animal hormone supplementation, results in the growth of the human smooth muscle cells out of the injection site where they acquire a blood supply through angiogenesis. Experiments to demonstrate the inhibitory effects of CCN5 overexpression on human uterine smooth muscle cells within this new *in vivo* system are underway. These data bring us one step closer to the realization of CCN5 as a potential therapeutic for multiple smooth muscle pathologies, including uterine fibroids.

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MICROVASCULAR PERICYTES EXPRESS CCN2 IN TISSUE FIBROSIS

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The cell and molecular mechanisms that link microvascular damage to the fibrogenic pathology in systemic sclerosis (SSc) are not fully understood. However, the endothelium-derived production of endothelin-1 (ET-1) and the fibroblast-derived production of CCN2 are likely to play key roles in this process. Dermal fibroblasts (DF; $n=6$) were obtained from control and SSc tissue. Pericytes were isolated from human placenta. Gene expression profiles of fibroblasts and pericytes was assessed by gene chip using the Affymetrix U133A gene chips and analyzed by D-Chip software in the presence or absence of ET-1 (100 nM). The effect of ET-1 the phenotype of normal, SSc fibroblasts and pericytes was also assessed by functional assays. Early cultured pericytes (passage < 4) expressed a-SMA and CCN2, but little or no expression of the fibroblast marker AS02 or collagen type I ($p<0.05$). In late passage pericytes (passage >5), AS02 and collagen type I production were significantly increased ($p<0.05$). ET-1 significantly stimulated pericytes and normal fibroblasts to produce CCN2 and collagen ($p<0.05$). These data strongly suggest that pericytes can acquire a fibrogenic phenotype suggesting that this cell type may be linked to the fibrosis in SSc. Thus pericytes represent an additional cell type that must be taken into account when considering pathogenic mechanisms and therapeutic targets in SSc.

EPITHELIAL AND CONNECTIVE TISSUE CELL CTGF/CCN2 EXPRESSION IN GINGIVAL FIBROSIS: ROLE OF EPITHELIAL-MESENCHYMAL TRANSITION

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Drug-induced gingival fibrosis is a common side effect of certain medications, and non-drug induced forms occur either as rare inherited or idiopathic gingival fibromatosis (GF). In the present study, we have investigated both the epithelial and connective tissue expression of CTGF/CCN2 in gingival fibrosis. Findings prompted us to investigate epithelial-mesenchymal transition (EMT), as a possible mechanism underlying these gingival pathologies. Gingival overgrowth samples were from subjects receiving phenytoin (PHE), nifedipine (NIF), or Cyclosporin A (CsA), or diagnosed with GF. Control tissues were from healthy donors. Connective tissue growth factor (CTGF/CCN2) expression in connective tissue fibroblasts was positively related with the degree of fibrosis. Cellular and extracellular CTGF/CCN2 content in PHE and GF tissues was significantly ($p<0.05$) higher (4-7-fold and 5-6-fold, respectively) compared to the other drug-induced gingival overgrowth tissues and the controls. Higher CTGF/CCN2 staining was accompanied by an increased abundance of fibroblasts and connective tissue fibers. CTGF/CCN2 was also highly expressed in the epithelium of fibrotic gingival tissues. This finding was confirmed by in situ hybridization. Real time PCR analyses of RNA extracted from control and drug-induced gingival overgrowth tissues for CTGF/CCN2 were fully consistent with these findings. Normal primary gingival epithelial cell cultures were next analyzed for the basal and TGF-β1—or lysophosphatidic-acid stimulated CTGF/CCN2 expression at the protein and RNA levels. Cultured epithelial cells express CTGF/CCN2, and TGF-β1 and lysophosphatidic acid each further stimulates CTGF/CCN2 expression, but with different kinetics. As an indication of EMT, the levels of E-cadherin, a specific marker of epithelial cells, were significantly lower in gingival fibrosis compared to control tissues while there was no detectable expression in fibroblasts. Meanwhile, epithelial Fsp-1, which is a marker of mesenchymal cells, showed a significant increase in oral epithelium of gingival fibrosis samples compared to

control tissues. These findings suggest a possible role for CTGF/CCN2 in promoting development of fibrotic lesions in human gingiva while interactions between epithelium and stroma in the form of EMT seem likely to contribute to gingival fibrosis.

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FIBROSIS IN DUCHENNE MUSCULAR DYSTROPHY; ROLE OF CCN2 AND ITS POTENTIAL INHIBITION BY DECORIN

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One of the features of Duchenne Muscular Dystrophy (DMD) is the progressive accumulation of extracellular matrix (ECM) in a process called fibrosis. In the murine model of DMD, the mdx mouse, fibrosis is quite evident in the diaphragm but less prominent in leg muscles such as tibialis anterior. Connective tissue growth factor (CCN2/CTGF) a pro-fibrotic growth factor has been implicated as one of the key mediators of fibrosis in many tissues. The exact role of CTGF in skeletal muscle fibrosis, as well as, its effect on myoblasts and myotubes is unknown. Mdx mice under exercise protocols (1 month, twice per week) shown an important augment in fibrosis, characterized by an increase in fibronectin, collagen type III and periostin, together with CTGF. Myoblasts and myotubes are able to synthesize CTGF in response to transforming growth factor type-b (TGF-b) and lysophosphatidic acid (LPA). In myoblasts, CTGF increase the amount of ECM molecules and down-regulates desmin and MyoD, inducing a loss of the commitment of this cell to the muscle cell lineage. Myoblasts null for the proteoglycan decorin, show increased basal level of ECM molecules and an enhanced response to CTGF compared to wild type myoblasts, suggesting an inhibitory effect of decorin on CTGF activity. In fact, the addition of soluble decorin caused an inhibition of CTGF activity in wild type myoblasts and fibroblasts. Furthermore, co-immunoprecipitation assays of purified CTGF and decorin indicate that both molecules directly interact. Interestingly, decorin is endocytosed, in myoblasts but not in myotubes through LDL receptor-related protein (LRP), a known receptor of CTGF. In summary, these results suggest that CTGF is implicated in skeletal muscle fibrosis, which myoblasts contribute to this fibrotic process. CTGF dedifferentiate myoblast, a repair cell source in DMD and, decorin inhibits CTGF fibrotic activity by direct interaction with the growth factor.

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TISSUE-SPECIFIC REGULATION OF CCN2/CTGF IN GINGIVAL FIBROBLASTS AND ITS RELATIONSHIP TO GINGIVAL FIBROSIS

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Connective tissue growth factor (CCN2/CTGF) is expressed at high levels in some forms of gingival overgrowth. Lesions in severe cases cover teeth and interfere with normal masticatory function and treatments include repeated surgical excisions. Regulation of CCN2/CTGF expression in human gingival fibroblasts is unique and is related to the tissue-specificity of this pathology. TGFβ1-stimulated expression of CCN2/CTGF in gingival fibroblasts is resistant to inhibition by PGE_2, whereas human lung and renal fibroblastic cells are highly sensitive. Resistance in gingival fibroblasts is conferred by two mechanisms: (1) activation of the EP3 prostanoid receptor that results in stimulation of Jun-N-terminal kinase (JNK1); and (2) a weak cAMP response and weak inhibition of JNK1 resulting from activation of the EP2 prostanoid receptor. Data show that JNK1 is the major MAP kinase required for the TGFβ1-induced expression of CTGF in gingival fibroblasts determined in studies employing pharmacologic inhibitors, and recombinant dominant-negative JNK1 adenovirus. Stimulation of adenylate cyclase with forskolin more significantly reduces JNK activation in response to TGFβ1 in gingival-compared to lung fibroblasts. Specific activation of the EP3 receptor with sulprostone enhances JNK1 activation in gingival fibroblasts. Thus, EP3 activation by PGE_2 is a mechanism by which gingival cells overcome the inhibition of CTGF expression caused by the stimulation of cAMP accumulation in response to stimulation of the EP2 prostanoid receptor or forskolin. TGFβ1-induced expression of CCN2/CTGF in gingival fibroblasts is independent of the activation of the small GTPase, RhoA. While RhoA is not involved in mediating the TGFβ1-stimulated expression of CCN2/CTGF in gingival fibroblasts, Rho-family GTPases Rac1 and Cdc42 are. This was demonstrated by overexpression of recombinant dominant-negative adenoviral forms of RhoA, Rac1 and Cdc42 in gingival fibroblasts, and effects of TGF-β1 regulation of CCN2/CTGF determined. Results were independently confirmed with pull-down assays for activated RhoA, in which LPA stimulation of gingival fibroblasts resulted in detecting activated RhoA, whereas TGF-β1 did not activate RhoA. These findings have permitted us to propose a potential treatment strategy to block CCN2/CTGF expression in gingiva that independently targets two complementary pathways. The combination of the HMG-CoA reductase inhibitor lovastatin, which blocks the activation of small GTPases, and the adenylate cyclase activator, forskolin (a JNK inhibitor in gingival cells), together reduce TGF-β1 stimulated CCN2/CTGF protein levels to unstimulated levels. Additional studies in progress are furthering our understanding of unique aspects of CCN2/CTGF regulation that may similarly be potentially addressed by pharmacologic approaches. Supported by NIH grants R01 DE11004, M01 RR00533, and K08 DE016609.

CCN3 (NOV) IS A NEGATIVE REGULATOR OF CCN2 (CTGF) AND A NOVEL ENDOGENOUS INHIBITOR OF FIBROSIS IN EXPERIMENTAL NEPHROPATHY

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Fibrosis is a major cause of end stage renal disease (ESRD), and although factors responsible for its initiation are being elucidated, a lack of understanding of the downstream regulatory pathways has prevented development of specific anti-fibrotic therapies. CCN2 (CTGF) has emerged as a critical molecule acting downstream of TGF-b to drive fibrosis, making it a new therapeutic target. However, suppression of CCN2 has been difficult. We examined the possibility that CCN3 (NOV), another CCN family member with different reported biological activities, might act as an endogenous negative regulator of CCN2 with the capacity to limit the fibrotic response, including the overproduction of extracellular matrix (ECM). We demonstrate for the first time, using an in vitro model of renal fibrosis, that both exogenous treatment and transfection with the over-expression of the CCN3 gene, in mesangial cells markedly down-regulates CCN2 activity and blocks ECM over-accumulation stimulated by TGF-b. Conversely, TGF-b treatment reduces endogenous CCN3 expression and increases CCN2 activity

and matrix accumulation, indicating an important, novel yin / yang effect. Preliminary animal studies support these in vitro findings. CCN3 then, as a negative regulatory molecule of CCN2 and the effects of TGF-b, may act naturally to limit fibrosis *in vivo*, and therefore provide opportunities for novel endogenous-based therapy.

TARGETED CCN2 GENE THERAPY IN EXPERIMENTAL LIVER FIBROSIS

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Hepatic stellate cells (HSC) are a relatively minor and normally quiescent cell population in the liver that reside in the space of Disse and which, following injury, become "activated" into α-smooth muscle actin (αSMA)-expressing myofibroblastic cells. Upon activation, HSC are responsible for deposition of excess scar tissue through their production of collagen types I and III, proteoglycans, fibronectin, laminin and activation of tissue inhibitors of matrix metalloproteases which prevents fibrolysis by inhibiting matrix metalloprotease activity.

Recent data have firmly established that CCN2 is an important player in HSC biology. CCN2 is produced by HSC either as a function of activation or in response to stimulation of the cells by pro-fibrotic molecules or growth factors. CCN2 mRNA and protein are increasingly expressed during progressive activation of cultured primary rat HSC, or in response of the cells to stimulation by TGF-β, VEGF, lipid peroxidation products, acetaldehyde or PDGF-BB. The CCN2 receptor, integrin αvβ3, is strongly upregulated during HSC activation allowing CCN2 to drive adhesion, migration, proliferation, and fibrogenesis in activated cells but not their quiescent counterparts. CCN2 is also a survival factor for activated HSC.

These data show that CCN2 drives both fibrogenic and anti-apoptotic pathways in HSC and reinforce the notion that CCN2 is a realistic therapeutic target in liver fibrosis. To address this question directly, we developed a strategy to specifically target CCN2 production in activated HSC *in vivo*, as tested in mouse models of hepatic fibrosis. Modified liposomes containing CCN2 siRNA were coated with a synthetic peptide that was designed to home specifically to activated HSC i*n vivo*. Balb/c mice received daily injections of CCl_4 or oil control for 3 weeks (preventative model) or 5 weeks (curative model) and, for the last 2 weeks of each regimen, some mice also received daily treatments of CTGF siRNA in the targeted liposomes. Hepatic mRNA or protein levels of key fibrotic markers (CCN2, TGF-β, αSMA, collagen 1) were assessed and showed that in both preventative and curative models, the targeted liposomes were completely effective as an anti-fibrotic therapy, and were significantly more efficacious than their non-targeted counterparts.

These data (i) show that attenuation of CCN2 production in activated HSC in fibrosing liver is an effective anti-fibrotic strategy and (ii) demonstrate the utility of a targeted HSC approach as a means of delivering and testing therapeutic agents.

CELL TYPE-SPECIFIC REGULATION OF CONNECTIVE TISSUE GROWTH FACTOR BY HYPOXIA

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Connective tissue growth factor (CTGF) is a matricelluar protein which interacts with VEGF and thus modulates angiogenesis. Hypoxia, a major stimulus of angiogenesis in tumor tissue, has been connected to the induction of CTGF. However, upregulation of CTGF was not consistently observed. Therefore, the aim of the present study was to investigate the molecular mechanisms of hypoxia-induced regulation of CTGF.

Expression of CTGF was investigated in various cell lines, particularly in established microvascular endothelial cell lines (glEND.2, HMEC) and human proximal tubular epithelial cell lines, (HK-2, HKC8) as well as human primary tubular cells. Cells were exposed to hypoxia or stimulated with dimethyloxalylglycine (DMOG), which leads to the stabilization of the hypoxia-inducible factor HIF-1a. Upregulation of CTGF was observed in human and mouse endothelial cells, whereas no change or down-regulation was detectable in tubular epithelial cells. DMOG-mediated stabilization of HIF-1a was linked to CTGF expression in endothelial cells, because siRNA against HIF-1a reduced CTGF expression. However, HIF-1*a* was stabilized in all cell types exposed to DMOG suggesting the existence of cell-type specific co-regulators.

Several signaling pathways, which have previously been shown to be relevant for CTGF induction, were not involved in hypoxia-induced CTGF expression (MAPkinases p42/44 and p38, or RhoA-associated kinase). Inhibition of PI3-kinase-AKT signaling by LY294002, however, strongly induced basal and DMOG-stimulated CTGF expression in endothelial cells, whereas in epithelial cells, CTGF was slightly downregulated.

The kinase AKT negatively regulates transcription factors of the FoxO family, which are active in their dephosphorylated form. FoxO 1/3 siRNA significantly reduced induction of CTGF by LY294002 in endothelial cells, establishing FoxO proteins as downstream mediators of AKT, relevant for the induction of CTGF. Furthermore, hypoxia– and DMOG-induced CTGF expression was also reduced by FoxO 1/3 siRNA, suggesting a connection between HIF-1a signaling and regulation of FoxO 1/3 activity.

FoxO transcription factors have been previously described as context-dependent regulators of cell growth and differentiation. In this study, we were able to link these transcription factors to the cell type-specific regulation of CTGF and demonstrate their relevance for the differential regulation of CTGF by hypoxia, the functional implications of which need further consideration.

ELASTIN RECEPTOR SUBUNITS FACILITATE ELASTIC FIBER ASSEMBLY AND MODULATE CELLULAR PROLIFERATION

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We have discovered that the elastin-binding protein, which is identical to the spliced variant of ß-galactosidase, forms a cell-surface-targeted complex with two proteins considered as "classic lysosomal enzymes," protective protein/cathepsin A (PPCA) and neuraminidase-1 (Neu1). This recyclable complex can also bind intracellular tropoelastin and serve as a molecular chaperone for this precursor of elastin.

We have also establish that transgenic mice deficient either in Neu1 or PPCA do not assembly normal elastic fibers. This observation, and additional results from extensive *in vitro* studies lead to the conclusion that the cell-surface-residing that PPCA proteolytically activates Neu1, which in turn desialylates neighboring microfibrillar glycoproteins and facilitate the deposition of insoluble elastin. Recently, we also introduced a novel mechanism in which Neu1 may limits proliferation of human arterial smooth muscle cells (SMC) and skin fibroblasts by desialylating their cell membrane-residing sialoglycoproteins that directly propagate

mitogenic signals. We demonstrated that treatment of these cells with the neuraminidase inhibitor or with anti-Neu1 antibody induced significant up regulation in their proliferation in response to the growth factors-rich fetal serum. Conversely, treatment with *Clostridium perfringens* neuraminidase (which is highly homologous to Neu1) decreased SMC's proliferation. We further found that pretreatment of SMCs and skin fibroblasts with neuraminidase abolished their mitogenic response to recombinant PDGF-BB and IGF-II, and that fibroblasts derived from Sialidosis patients (which are exclusively deficient in Neu1) were more responsive to PDGF-BB and IGF-II than normal fibroblasts. Furthermore, we provided evidence that neuraminidase caused desialylation of the PDGF–and IGF-1 receptors and diminished intracellular signals induced by their mitogenic ligands, PDGF-BB and IGF-II.

The most recent data indicate that Neu1 also desialylates the cell surface insulin receptor (IR) on skeletal cells myoblasts (L6WT). However, in contrast to an inhibitory effect on cellular proliferation observed after desialylation of IGF-1R or PDGFR, the removal of sialic acids from IR of L6WT cells leads to a more potent metabolic and proliferative response to physiological dose (10 nM) of insulin.

We also found that PPCA-deficient mice, which in addition to Galactosialidosis phenotype are characterized with arterial hypertension, demonstrate significantly heightened levels of Endothelin-1 (ET-1). Importantly, we also provided evidence that cell surface-residing PPCA plays a non redundant role in the regulation of blood pressure through proteolytic inactivation of a potent vasoconstrictor and mitogen, ET-1.

ABNORMAL EXTRACELLULAR MATRICES IN RARE AND COMMON DISORDERS

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Many of the genes required for the normal structure and function of the connective tissues have been identified from the study of rare genetic disorders. Many of the genes encode macromolecules of the extracellular matrices but others are involved in cell signalling, cell adhesion and cell-matrix interactions. Examples include the fibrillar and other collagens, thrombospondins such as cartilage oligomeric matrix protein, and matrilins such as matrilin 3. The combination of protein studies using cell cultures from affected patients as well as tissues and cultures from animal models have provided valuable insights into the structural and functional consequences of mutations. In patients with lethal or very severe phenotypes the mutant gene effect sizes are overwhelming so that recurrent mutations can be expected to be yield similarly severe phenotypes. For example, glycine substitutions within the triple helical domain of the type I collagen chains in lethal forms of osteogenesis imperfecta severely impair the assembly, modifications and secretion of type I collagen molecules. The extracellular matrices are very abnormal. The type I collagen producing cells undergo an endoplasmic reticulum stress survival response, impaired differentiation and apoptosis. In contrast, genetically determined connective tissue disorders that become clinically evident in late childhood and in adulthood are usually milder and show more phenotypic variability than those that manifest early. An example, is the common form of osteogenesis imperfecta which is associated with COL1A1 haploinsufficiency and highly variable skeletal fragility. In such individuals, the mutant COL1A1 gene effect size is likely to be less than for COL1A1 mutations that produce lethal phenotypes. It is also likely that the variable skeletal fragility is due to the differing genetic backgrounds and environmental influences amongst different family members that bear the same COL1A1 mutation. Currently, little is known about the genes and environmental factors that determine the susceptibility or resistance to COL1A1 haploinsufficiency. Studies of various bone health parameters, such as peak bone mass, in man and in inbred mice have shown that most traits are determined by many gene variants that each exert very small effects on the phenotype of interest. These various findings suggest that the normal development, maintenance and repair of tissues such as bone and cartilage are controlled by sets of 'bone health' and 'cartilage health' genes that act in networks. The relative contribution of various genes and their protein products may vary throughout life. The health of the tissue is likely to be largely determined by the genetic variants that an individual bears. Novel strategies are now available to define the gene and protein networks and to identify the patterns that are associated with the onset of connective tissue disorders at various ages.

CELL AND GENE THERAPY PRESERVES MATRIX HOMEOSTASIS: A NOVEL PARACRINE MECHANISM

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Current attempts to regenerate the damaged myocardium after a myocardial infarction have focused on therapies directed at increasing regional perfusion and salvaging viable cardiomyocytes. Accumulating evidence suggests that implanting healthy muscle cells into the damaged myocardium can prevent infarct thinning and chamber dilatation. Cell transplantation has been proposed to encourage the recruitment of stem cells from the bone marrow or the heart to repopulate the infarcted region. Within the myocardial scar tissue, these neo-myogenic cells can prevent ventricular dilatation and delay the onset of cardiac dysfunction. Early clinical trials suggest encouraging results for cell therapy.

Although the favourable effects of cell therapy on post-infarction myocardial regeneration have been observed, the underlying mechanisms have not yet been elucidated. For example, while stimulating neo-vessel formation and muscle cell engraftment within the scar may contribute to enhanced regional and global function, the number of cells surviving implantation is too small to alone account for these functional benefits.

An alternative explanation may involve the effect of the engrafted cells on remodeling of the extracellular matrix, which is initiated following a myocardial infarction by the imbalance between matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs). Matrix remodeling is recognized as a central process underlying the maladaptive reorganization of cardiac size, shape, and function that determines the progression of heart failure. Disruption of the matrix network may contribute to cardiomyocyte apoptosis, leading to chamber dilatation. We have demonstrated that cell transplantation can prevent scar thinning and cardiac dilatation after an infarction by altering the response of the matrix or restoring its structure. We found that smooth muscle cells implanted into animal hearts survived and altered matrix remodeling both within and remote from the region of implantation. We also observed decreased matrix metalloproteinase (MMP-2 and -9) activities in transplanted compared to control hearts. The results were maintenance of the matrix structure, and prevention of ventricular dilatation.

To enhance cell therapy-induced matrix preservation by regulating MMP inhibition in the infarcted and remote myocardium, we implanted the infarcted heart with cells genetically modified to over-express TIMP-3. These studies established the proof of concept for cell-based gene therapy as an effective, clinically-relevant approach to matrix modulation. We also identified an intricate dose–and time-dependant effect of TIMP-3 over-expression in the modulation of cardiac remodeling. Our results highlight the efficacy of cell-based gene delivery systems and the synergistic benefit of gene and cell therapies used to target matrix remodeling after a myocardial infarction.

Overall, the data suggest that implanted cells prevent ventricular dilatation through an alteration of matrix metabolism, which is a possible mechanism by which cell transplantation improves heart function.

MT1-MMP AND CONTRACTILITY PROMOTE INVASIVE BEHAVIOUR BY OVARIAN CANCER CELLS

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Cell- matrix interactions modulate cell fate, polarity, motility, survival and invasion in both physiological and pathological processes. Ovarian cancer, the most lethal gynaecological cancer, is typically detected at a late/advanced stage subsequent to metastasis within the peritoneal cavity. Since cell motility and degradation are important contributors to tissue invasion, it is critical to use *in vitro* assay systems that best reflect the circumstances *in vivo*. Evaluation of Matrigel and collagen I matrices as representatives of basement membrane and stromal matrix barriers respectively, revealed the inadequacy of Matrigel, which cells penetrated in absence of matrix metalloprotease (MMP)-mediated matrix degradation. MT1-MMP (MMP-14), a transmembrane MMP with potent pericellular proteolytic activity, was determined to be a critical mediator of collagen I matrix degradation and invasion by ovarian cancer cells, and its ectopic expression conferred an invasive ability to previously non-invasive cell lines. Although cells that expressed MT1-MMP were generally more motile, MT1-MMP did not promote cell motility. Proteomic profiling of the cell lines was therefore performed to reveal factors contributing to the divergent motile capacities. Several proteases, integrin subunits, and proteins that mediate actin cytoskeletal dynamics were upregulated in the motile cell lines, supporting the importance of cell-matrix interactions in stimulating this behaviour.

Within peritoneal ascites fluid, ovarian cancer cells exist both individually and as multicellular spheroid aggregates. Spheroid aggregates contain abundant matrix and the constituent cells have enhanced resistance to radiation and many chemotherapeutics. A striking correlation was evident between the ability of the cells to form compact spheroids and their capacity to invade/penetrate in a 3D culture system. These data suggest that behaviours possessed by the invasive cell lines promote their spheroid formation such that an aggressive cancer cell subpopulation may acquire preferential resistance to chemotherapeutics. Intriguingly, in this 3D model, although MMP activity was required for invasion, additional factors related to cell motility appeared to limit spheroid cell dissemination. Contractile behaviour and a mesenchymal phenotype were characteristics common to the invasive, compact spheroid forming cell lines. Furthermore, preliminary data indicate CCN proteins, particularly CTGF and NOV, to be upregulated in the 3D-invasive, compact spheroid-forming subset of cell lines. The role of MT1-MMP and contractile behaviour as potential contributors to ovarian cancer metastasis will be discussed, as well as issues relating to the use of collagen I and Matrigel as invasion matrices in 2D transwell and 3D invasion systems.

CCN3 PROMOTES MELANOMA PROGRESSION BY REGULATING INTEGRIN EXPRESSION, ADHESION AND CHEMORESISTANCE

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Overexpression of CCN3 was detected in metastatic melanoma cells as compared to cells of the primary tumor from the same patient. Analysis of CCN3 in short-term cultures from 50 melanoma lesions revealed a heterogeneous expression of the 46-kDa full-length protein and the 32-kDa truncated form. In fact, some melanomas were negative for CCN3 expression, while others expressed both isoforms at the cellular level and secreted in the culture medium. Other melanomas displayed only the full-length or only the short isoform with or without protein secretion. The different protein expression patterns were not associated with gene alterations or polymorphisms. Cell fractions and immunofluorescence analysis demonstrated that the 46-kDa protein has a prevalent cytoplasmic localization, while the 32-kDa form has a nuclear localization and lacked the N-terminal domain.

Similarly to metastatic cells expressing high levels of CCN3, cells transfected to overexpress CCN3, despite a reduction in cell proliferation, showed increased adhesion to ECM proteins, particularly laminin and vitronectin, while inhibition of CCN3 expression by siRNA decreased adhesion. CCN3 overexpression increased expression of laminin and vitronectin integrin receptors $\alpha 7\beta 1$ and $\alpha v\beta 5$ by increasing their mRNA production. Moreover, CCN3 secreted by melanoma cells acted as an adhesion matrix protein for melanoma cells themselves.

Immunohistochemistry performed on melanoma specimens from which the cell lines were derived confirmed that the different levels of expression occurring *in vivo* are maintained in cultured cells. Analysis of CCN3 protein expression with respect to melanoma progression detected the protein in all visceral metastases tested and in most nodal metastases from relapsing patients, but only in a few nodal metastases from non-relapsing patients and cutaneous metastases. Consistently, xenotrasplantation in immunodeficient mice showed a higher metastatic potential of melanoma cells overexpressing CCN3. CCN3-transfected cells showed a higher resistance to apoptosis-induced by treatment with cytotoxic drugs. Taken together, these data indicate a role for CCN3 in melanoma cell interaction with the ECM by regulating integrin expression, resulting in altered cell adhesion and increased chemoresistance, leading melanoma progression to aggressive disease.

CCN3 RESTORES GROWTH REGULATION IN CHRONIC MYELOID LEUKAEMIA

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Chronic Myeloid Leukaemia (CML) is characterized by expression of the constitutively active Bcr-Abl tyrosine kinase. We have shown previously that the negative growth regulator, CCN3, is down-regulated as a result of Bcr-Abl kinase activity and that CCN3 has a reciprocal relationship of expression with *BCR-ABL* (McCallum et al, Blood 2006; 108(5):1716–23). We now show that CCN3 confers growth regulation in CML cells by causing growth inhibition and apoptosis.

To further investigate CCN3 mode of growth regulation, K562 CML cells were either transfected with CCN3 or treated with recombinant CCN3 (rCCN3, Peprotech, UK). K562 cells were transfected with CCN3 or empty vector (EV) using amaxa nucleofector technology (Amaxa GmbH). Increased CCN3 expression significantly reduced colony formation by 65.4%±18.8 when compared to cells transfected with vector alone ($p=0.027$, $n=3$). Flow cytometry identified an increased accumulation of cells within the $subG_0$ area as a consequence of CCN3 expression (Mean $subG_0$ for CCN3 21.8%±0.7 and EV 9.9%±4.6% respectively, $p=0.027$, $n=3$). In addition, increased levels of CCN3 caused decreased phosphorylation of ERK2 and increased levels of cleaved caspase 3.

K562 cells were treated with and without recombinant CCN3 (rCCN3) for 24 h and either plated in methyl cellulose culture or analysed by flow cytometry and Western blotting. rCCN3 reduced the colony formation capacity of K562 cells by 14%±2.8 in comparison to untreated control (100%±2.3, p=0.015, n=3) and caused an increased accumulation of cells within the subG$_0$ area of cell cycle (Mean subG$_0$ for rCCN3 treated cells 23.7%±6.4 and untreated control 9.3%±3.8, p= 0.014, n=3). rCCN3 treatment also resulted in decreased phosphorylation of ERK2 and an increase in levels of cleaved caspase 3.

To determine if primary human CML cells were responsive to CCN3, CD34+ cells were extracted from CML peripheral blood patient samples at diagnosis and treated with rCCN3 (range 1 nM to 10 pM) for 24 h prior to plating in methyl cellulose cultures. Primary CD34+ CML cells treated with rCCN3 showed dose dependent inhibition of colony formation (19.5%±4.0 (p=0.001), 17.8%±2.7(p=0.003) and 16.6%±4.6 (p=0.003) for 1 nM, 100 pM and 10 pM respectively, n=3).

CCN3 restores growth regulation in CML cells by inhibiting cellular growth pathways and inducing apoptosis. Restoration of CCN3 levels in CML cells may provide an additional therapeutic strategy in the management of CML.

PROGNOSTIC ROLE OF CCN3 IN OSTESARCOMA AND EWING'S SARCOMA

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Osteosarcoma and Ewing's sarcoma, the two most common bone tumors, still lack prognostic markers that could distinguish patients before therapy and drive treatment choices. We assessed the prognostic value of CCN1-3 genes, involved in fundamental biological processes.=Expression of CCN1-3 was measured by either at gene and protein level by microarray techniques or quantitative PCR and by immunohistochemistry in 45 diagnosed localized tumors. Cancer specific survival was estimated using the Kaplan-Meier method. In addition we evalutated possible associations with osteoblastic differentiation. While CCN3 is barely expressed in normal proliferating osteoblasts and mesenchymal stem cells, its expression was generally high in osteosarcoma and its level of expression did not correlate with any specific osteoblastic differentiation genes. High expression of CCN3 significantly correlated with worse prognosis in osteosarcoma. This may be only partly explained by the association with the expression of MRP1 and MRP4, two ABC transporters that also acted as predictors of worse outcome in our study. In Ewing's sarcoma we confirmed the same type of association. High expression of CCN3 was associated with worse prognosis and higher metastatic risk. Taking advantage of antibodies that recognize the different domains of CCN we found that a high percentage of cases expressed a variant type of CCN3 lacking the NH3 domain. Lack of this domain was also associated with worse prognosis in patients treated with chemotherapy and radiotherapy. Thus, we found that assessment for CCN3 expression levels at diagnosis may represent a useful molecular tool to early identification of sarcoma patients with different prognosis.

CCN1/CYR61 ENHANCES THE METASTATIC POTENTIAL OF HUMAN OSTEOSARCOMA CELL LINES AND ACTIVATES THE AKT PATHWAY

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Osteosarcoma is the most frequent primary malignant bone tumor in childhood with a high propensity for metastasis. Despite significantly improved treatment strategies through the use of combined chemotherapy and surgery, patients with metastatic or recurrent disease continue to have a poor prognosis.

Recently, CCN1/Cyr61 emerged as a multifunctional protein that also stimulates angiogenesis and tumor growth. Aberrant expression of CCN1/Cyr61 is associated with several patho-physiological processes including tumorigenesis.

In this study, we demonstrated that CCN1/Cyr61 expression is upregulated in highly metastatic human osteosarcoma cell lines, as well as in primary tumor tissues of osteosarcoma patients. Moreover, overexpression of CCN1/Cyr61 in osteosarcoma cell lines with low metastatic potential enhanced their metastatic activity in-vitro.

In addition, we demonstrated that CCN1/Cyr61 activates AKT in a specific manner and leads to the translocation of the cell cycle inhibitor p21 from the nucleus to the cytosol. Thus, our data suggest a role of CCN1/Cyr61 in the regulation of the metastatic process in osteosarcoma which is likely mediated, at least in part, by the stimulation of the AKT signaling and the cell cycle control.

CCN3 SUPPRESSES GROWTH AND INDUCES ACTIN CYTOSKELETAL REORGANIZATION IN BREAST CANCER CELLS

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Several lines of evidence, including a compilation from gene expression analyses of cancer tumor arrays, demonstrate that the expression of CCN3 is lower in higher-grade breast tumors. Indeed, CCN3 has been shown to inhibit growth and proliferation in many cell types, and its absence in high grade tumors is therefore not surprising. Interestingly, recent data has also shown that gap junction protein connexin43 (Cx43), whose expression is similarly downregulated in aggressive breast tumors, upregulates the expression of CCN3 in gliomas. We therefore investigate whether 1) CCN3 negatively regulates growth in breast cancer cells and 2) CCN3 as a possible downstream signaling mediator of Cx43-dependent growth control. We performed gain/loss of function studies in 2 breast cell lines—Hs578T cells that expressed high levels of Cx43 and CCN3, and MDA-MB-231 cells with low endogenous Cx43 and CCN3. Using standard growth proliferation and migration assays, we determined that overexpression of CCN3 in MDA-MB-231 cells reduced growth but had little effect on directed cell migration. Further analysis showed that CCN3 induced the formation of multiple actin-rich pseudopodia. Using Cx43 shRNA to knock down Cx43 protein in Hs578T cells, we observed a corresponding decrease in CCN3 expression, indicating Cx43 is able to regulate CCN3 expression. On the other hand, a reduction of CCN3 level by siRNA did not affect the expression and coupling of Cx43, suggesting CCN3 is acting downstream of Cx43. Cx43 are transmembrane proteins that can form channels to allow the exchange of materials between cells, or interaction with signaling molecules such as cell adhesion protein ZO-1 with its C-terminal tail. To further delineate the relationship between Cx43 and CCN3, we used co-immunoprecipitation assays to confirm that the C-terminal tail of Cx43 contains the CCN3-interacting sites. In addition, we added fluorescent- labeled CCN3 protein to the culture medium of Hs578T cells and observed the uptake and

localization of the labeled CCN3 as intracellular vesicles, suggesting that secreted CCN3 can be internalized by breast cancer cells. A small percentage of these vesicular CCN3 also appears to associate with Cx43. Our results suggest Cx43 may modulate cell growth by directly regulating the expression and localization of CCN3.

CCN1: A NEW TARGET FOR CHEMOTHERAPEUTICAL DRUGS IN BREAST CANCER TREATMENT

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The angiogenic factor CCN1, plays a key role in both the maintenance and the enhancement of a malignant phenotype in breast cancer. CCN1 is overexpressed in about 30% of triple negative breast carcinomas, whereas that in normal breast tissues are negligible. CCN1 expression is highly correlated with advanced disease. Women with advanced breast cancer often develop bone metastases with high CCN1 level expression. Recently, we have demonstrated that CCN1 overexpression render human breast cancer cells highly resistance to the microtubule-interfering agent paclitaxel (Taxol), a drug of choice for the treatment of metastatic breast cancer. Most of the patients with breast cancer will develop bone metastases and they will receive bisphosphonate treatment. Zoledronic acid, pharmaceutically known as Zometa (ZOL), a third generation aminobisphosphonate, inhibits bone resorption and might prevent development of new osteolytic lesions induced by tumor metastases. Our data demonstrate that CCN1 is a prime candidate to target for a large portion of triple negative breast carcinomas. On the basis of this consideration, since CCN1 gives a Taxol resistant phenotype and ZOL has an effect on metastatic breast carcinomas, we tested the effect of Taxol and ZOL in breast cancer cells overexpressing CCN1 to determine whether the latter is a target for these drugs. We have confirmed that expression of $\alpha_v\beta_3$, a CCN1 receptor, is markedly up-regulated in breast cancer cells expressing CCN1. Our most recent data demonstrate that functional blockade of $\alpha_v\beta_3$ with a synthetic chemical peptidomimetic based upon the $\alpha_v\beta_3$ the RGD (Arg-Gly-Asp) motif, is specifically cytotoxic towards CCN1-over-expressing breast cancer cells. Pharmacological interference of the CCN1/$\alpha_v\beta_3$ interaction restores Taxol efficacy. To verify that the sole interaction between CCN1/$\alpha_v\beta_3$ integrin promotes chemo-resistance, namely Taxane-resistance, we took advantage of a CCN1 mutant with a point mutation at the position 125 of the protein (D125A) and which lacks the ability to interact with $\alpha_v\beta_3$. The studies showed that while CCN1 wild type induced Taxol resistance of breast cancer cells, the CCN1 mutant resulted in cells more sensitive to Taxol. Since CCN1 is a secreted protein, we can envision that the identification of a CCN1/$\alpha_v\beta_3$ autocrine loop suggests that targeting CCN1/$\alpha_v\beta_3$ may simultaneously prevent breast cancer tumorigenesis, angiogenesis and chemoresistance. We also establish that ZOL has a direct effect on CCN1 expression at transcriptional and protein level in breast cancer cells. Remarkably, ZOL inhibited branching and morphogenesis in 3D in Matrigel in addition to anchorage independent growth of CCN1 overexpressing cells. Collectively, these data demonstrate for first time a specific effect of ZOL on a pro-angiogenic factor involved breast cancer metastasis. Our data represents a new path for CCN1/$\alpha_v\beta_3$ targeted therapy, and a novel molecular avenue in the management and of metastatic breast cancer.

SUPPRESSION OF INVASIVE FRONT BY CCN5/WISP-2 IN BREAST CANCER IS MEDIATED THROUGH SILENCING OF MICRO RNA-10B

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Metastatic cells are a subset of primary tumor cells that have acquired the ability to complete a multi-step metastatic cascade, including migration, dissemination, extravasation, and eventual proliferation at a discontinuous secondary site. Understanding the molecular biology of cancer metastasis may provide novel intervention strategies to control metastatic lesions, and to improve the quality of life for the patients with these advanced diseases. MicoRNAs are naturally occurring single-stranded RNA molecules that post-transcriptionally regulate the expression of target mRNA transcripts. Many of these target mRNA transcripts are involved in cell proliferation, differentiation and apoptosis, processes commonly altered during tumorigenesis. Recent findings have shown that microRNA-10 b (miR-10 b) is highly expressed in metastatic breast cancer cells and positively regulates cell migration and invasion. Expression of miR-10 b is induced by Twist, a transcription factor that binds to putative promoter of miR-10 b. Since our recent studies proposed that CCN5 is a two-faced cancer gene and may participate in preventing micro-invasion of breast cancer cells, we hypothesized that CCN5 may regulate the invasive front through the nullification of miR-10 b expression. We found that silencing of CCN5 in ER-positive noninvasive breast tumor cell upregulates the miR-10 b expression parallel with HIF-1a and Twist. Moreover, the studies also showed that enhanced migration of CCN5 nullified MCF-7 breast cancer cells can be repealed by anti-miR-10 b. Collectively, these studies suggest that miR-10b is one of the key players in CCN5/WISP-2-mediated regulation of micro-invasion. Furthermore, we anticipate that the suppression of miR-10 b expression by CCN5 may be regulated by HIF-1a-Twist signaling pathway

IDENTIFICATION OF CCN PROTEINS AS SUBSTRATES FOR KALLIKREIN-RELATED PEPTIDASES

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Human tissue kallikreins (KLKs or kallikrein-related peptidases) comprise a subgroup of 15 homologous secreted serine proteinases encoded by a multigene family located on chromosome 19q13.4. KLKs are expressed in a myriad of tissues and are associated with important diseased states including cancer, inflammation and neurodegeneration. In cancer, their expression is often correlated with patient prognosis. So far, experimental evidence indicates that KLKs might promote or inhibit cancer-cell growth, angiogenesis, invasion and metastasis through degradation of growth-factor-binding proteins, activation of other proteinases and proteinase-activated receptors, and cleavage of extracellular-matrix components. Despite increasing interest in elucidating

functions of KLKs, examination of KLK12 biological role has remained limited. In this study, we used a degradomic approach to identify potential substrates of this enzyme. MDA-MB-231 cells were treated either with KLK12 or vehicle control in serum-free medium. After 30 min of treatment, overlying media were collected and the protein content was analyzed by mass spectrometry (OrbitTrap; Thermo Scientific). CCN1 was specifically identified in the medium of treated cells, suggesting that KLK12 might cleave and release this protein from the cell surface. To test this hypothesis, we investigated whether KLK12 digests CCN1 *in vitro*. Recombinant CCN1 was treated with KLK12 for varying times and the fragmentation products were analyzed by SDS-PAGE and mass spectrometry (Maldi-TOF).Our results showed that KLK12 rapidly cleaves CCN1 releasing two peptides fragments of 20.5 and 19.3 kDa. Further *in vitro* studies revealed that all the members of the CCN family can be digested by KLK12 with variable but comparable efficiency. Then, we investigated whether CCN members could serve as substrates for other KLKs (KLK1, 5, 6, 11, 13 and 14). We found that CCN1 and CCN5 were more efficiently cleaved by KLK12 and KLK14. Experiments are currently in progress to determine whether KLK-dependent proteolysis of CCN proteins alters their functional properties.

THE CCN FAMILY MEMBER CCN6: INHIBITION OF CCN6 REGULATES E-CADHERIN EXPRESSION IN THE BREAST EPITHELIUM THROUGH UP-REGULATION OF SNAIL AND ZEB1

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Epithelial–mesenchymal transition (EMT) is an important process during development by which epithelial cells acquire mesenchymal, fibroblast-like properties and show reduced intercellular adhesion and increased motility. E-cadherin has a central role of EMT, and its loss in cancer is associated with de-differentiation, invasion, and metastasis. CCN family members are secreted proteins implicated in epithelial-stromal cross talks, important for development and cancer progression. We previously reported that expression of CCN6 (or WISP3, Wnt-induced secreted protein 3) was frequently down-regulated in the highly aggressive inflammatory breast cancer and that CCN6 has tumor suppressor functions in breast cancer.

The stable CCN6-dificient HME cell line was constructed using two strategies: small interfering RNA-CCN6 in pSilencer2.1-U6 hygro and short hairpin RNA in a lentiviral vector (pLKO.1). Cells were cultured and observed for EMT-likes changes. CCN6 knockdown cells and controls were subjected to Western blots for E-cadherin. Luciferase assays to determine the effect of CCN6 inhibition on the activity of the E-cadherin promoter were performed. Protein and mRNA levels of the E-cadherin transcriptional repressors Snail, Slug, Sip1 and Zeb1 was investigated by Western blot and real-ime RT-PCR. CCN6-dificient HME cells were treated with siRNA-Snail or siRNA-Zeb1.

CCN6 inhibition in HME cells caused EMT approximately 7 days after stable transfection. This was accompanied by E-cadherin protein and mRNA down-regulation, with decreased activity of the E-cadherin promoter. By real-time RT-PCR, CCN6 inhibition led to an increase in Zeb1 and Snail mRNAs, whereas there was no effect on the other E-cadherin transcriptional repressors. Treatment of CCN6 knockdown cells with either Snail or Zeb1 siRNAs rescued E-cadherin protein expression.

Our results suggest an important role of CCN6 in regulating E-cadherin expression through transcriptional mechanisms involving Snail and Zeb1. We are now focused on the precise molecular mechanism by which CCN6 regulates Snail and Zeb1 during EMT.

THE CCN1/$\alpha_6\beta_1$ CONNECTION AND THE HORMONAL RESPONSE IN BREAST CANCER

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CCN1 plays a key role in both the maintenance and the enhancement of a malignant phenotype in breast cancer. The expression of CCN1 is sufficient to promote the acquisition of estrogen-independent and antiestrogens-resistant phenotype in breast cancer cells, apparently through a $\alpha_v\beta_3$-integrin. However, $\alpha_6\beta_1$-integrin is also associated with invasive carcinoma, although its specific participation in the hormonal response is unknown. Here, we investigated whether: 1) Expression of a CCN1 mutant with substitutions in the $\alpha_6\beta_1$-binding domains (TM) impedes CCN1's ability to induce an E_2-independent and/or the antiestrogens resistant phenotype, 2). CCN1 participates in the regulation of Estrogen Receptor-transcriptional activity in MCF-7 cells and 3). CCN1 mutant impairs the ability of the cells to growth in 3D-Matrigel culture. MCF-7 cell lines stably expressing either the CCN1-wild type (wt), CCN1-TM mutant or MCF-7/pBabe (empty vector) were growth in E_2 depleted conditions and subjected to anchorage-independent growth assays in the presence of estrogen (E_2 (10^{-9} M) and antiestrogens [Tamoxifen (10^{-7} M) and ICI 182,780 (10^{-7} M)]. Our results show that CCN1-induced anchorage-independent growth in the absence of E_2 and in the presence of antiestrogens. In contrast, MCF-7/TM mutation impaired this ability. These data suggest that the CCN1-$\alpha_6\beta_1$ interaction contributes to the CCN1-mediated induction of E_2 independent and antiestrogens resistant phenotype. To determine whether CCN1/$\alpha_6\beta_1$ interaction regulates the ER-transcriptional activity, we used estrogen-response-element (ERE)-reporter assays. E_2-deprived cells were transfected with ERE-Luciferase. Then cells were incubated with the treatments described previously and the Luciferase activity was detected. Results showed that MCF-7/CCN1 cells exhibited a higher basal ERE-Luc activity and in response to E_2 and antiestrogens than MCF-7 and MCF-7/TM cells. These data show that blockage of the CCN1/$\alpha_6\beta_1$ interaction abolishes the transcriptional activation of ER, suggesting a genomic role for these proteins in MCF-7 cells. The 3D-matrigel culture showed that MCF-7/TM cells growth less than MCF-7/CCN1 cells in the presence of E_2 confirming the previous results. Together these results support a possible nuclear role for CCN1 as a co-activator of ER, involved in the transcriptional activation of proliferative and survival ERE-genes in breast cancer cells.

NOVEL TRANSCRIPTIONAL REGULATION OF CCN2/CTGF BY NUCLEAR TRANSLOCATED MMP3

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CCN2/CTGF, designated from Connective Tissue Growth Factor, is a crucial regulator ofextra-cellular matrix (ECM), which promotes ECM synthesis and stabilization.

As the family name clearly implies, matrix metalloproteases (MMPs)are also localized to the ECM, where they function as proteases, modulating cell signaling by cleaving proteins such as matrix proteins, growth factors and growth factor receptors. We previously reported that strong expression of CCN2/CTGF in chondrocytic cells is through transcription enhancer dominant in chondrocytes (TRENDIC).

In this workshop, we report that matrix metalloprotease-3 (MMP3) is a novel TRENDIC-binding transcription factor for CCN2/CTGF expression. First, MMP3 cDNA was cloned as a TRENDIC-binding factor by southwestern screening. An interaction between MMP3 and TRENDIC was confirmed by a gel shift assay and chromatin immunoprecipitation. The CCN2/CTGF promoter was activated by transfected MMP3, whereas a TRENDIC mutant of the promoter lost the response. Also, the knock-down of MMP3 suppressed CCN2/CTGF expression. By cytochemical and histochemical analyses, MMP3 was detected in the nuclei of chondrocytic cells in culture and also in the nuclei of normal and osteoarthritic chondrocytes in vivo. The nuclear translocation of externally added recombinant MMP3 was observed in 30 min after the addition, and six putative nuclear localization signals in MMP3 were also found. Furthermore, we determined that heterochromatin protein γ coordinately regulates CCN2/CTGF by interacting with MMP3. These results indicated a novel trans-activation mechanism of CCN2/CTGF by the nuclear translocated MMP3 through binding with TRENDIC in chondrocytes. Although MMPs historically had been recognized as a protease for extra-cellular proteins, this study indicated that it is also a promoter of ECM synthesis through CCN2/CTGF trans-activation. This novel regulatory role of ECM may contribute to understanding the mechanism of not only development of cartilage, but also pathogenesis of arthritis and fibrosis.

INTEGRIN-MEDIATED MATRIX SIGNALING IN CELL DEATH AND SURVIVAL

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Regulated expression of the matricellular protein CCN1 (CYR61) is essential for cardiovascular development, and is associated with inflammation and tissue repair. During embryogenesis, CCN1 is critical for the survival of arterial vascular cells and mesenchymal cells of the cardiac cushion tissue, and *Ccn1*-null mice suffer aberrant apoptosis in these cell types (Mo *et al*., *Mol. Cell. Biol*. 22: 8709–8720; *Circ. Res.* 99: 961–969). CCN1 is an ECM-associated protein that supports cell adhesion and spreading through interaction with specific integrin receptors, and cell adhesion to CCN1 can promote survival in specific cell types such as vascular endothelial cells. However, in contrast to other matrix cell adhesive proteins, CCN1 can also induce apoptosis in other cell types while supporting cell adhesion and cell spreading (Todorović *et al.*, *J. Cell Biol.* 171: 559–568). Thus, CCN1 can promote cell survival or cell death in a cell type specific manner. Unexpectedly, CCN1 also acts as a powerful regulator of the cytotoxicity of inflammatory cytokines such as tumor necrosis factor a (TNFα)(Chen et al., *EMBO J.* 26:1257–1267). TNFα regulates inflammation and immunity, but also induces cell death in a context-dependent manner. Although TNFα is cytotoxic to certain tumor cell lines, it triggers apoptosis in normal cells only when NFκB-signaling or protein synthesis is blocked. We show that CCN1 can unmask the cytotoxic potential of TNFα without perturbation of NFκB-signaling or *de novo* protein synthesis, leading to rapid apoptosis in the otherwise resistant primary human fibroblasts. CCN1 acts through binding to integrins $\alpha_v\beta_5$, $\alpha_6\beta_1$, and syndecan-4, triggering the generation of reactive oxygen species (ROS) through a Rac1-dependent mechanism via 5-lipoxygenase and the mitochondria, leading to the biphasic activation of JNK necessary for apoptosis. In contrast to ROS generation and apoptosis induced by TNFα in the presence of cycloheximide, NADPH oxidase is not required for apoptosis induced by CCN1/TNFα. Furthermore, mice with the genomic *Ccn1* locus replaced with an apoptosis-defective *Ccn1* allele are substantially resistant to TNFα-induced apoptosis *in vivo*. These results indicate that CCN1 is a physiologic regulator of TNFα cytotoxicity, providing the contextual cues from the extracellular matrix for TNFα-mediated cell death.

INDUCTION OF CTGF BY TGF-B1 IN OSTEOBLASTS: INDEPENDENT EFFECTS OF SRC AND ERK ON SMAD SIGNALING

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Connective tissue growth factor (CTGF/CCN2) is a cysteine rich, extracellular matrix protein that acts as an anabolic growth factor to regulate osteoblast differentiation and function. In osteoblasts, CTGF is induced by transforming growth factor beta 1(TGF-β1) where it acts as a downstream mediator of TGF-β1 induced matrix production. The molecular mechanisms that control CTGF induction by TGF-β1 in osteoblasts are not understood. We have previously demonstrated the requirement of Src, Erk and Smad signaling for CTGF induction by TGF-β1 in osteoblasts. However, the potential interaction among these signaling pathways in osteoblasts has not been examined. In this study we demonstrate that TGF-β1 activates Src kinase in osteoblasts (primary rat and rat osteosarcoma cell line) and that treatment with the Src family kinase inhibitor, PP2, or two independent Src kinase mutants (kinase-dead; kinase-dead and open) prevented Src activation and CTGF induction by TGF-β1. The inhibition of Src kinase activity prevented TGF-β1 induced Smad 2 & 3 activation and Smad nuclear translocation in osteoblasts as determined by immunofluorescent and Western blot analyses. In addition, inhibiting Src prevented Erk activation. MAPKs such as Erk can modulate the Smad pathway through directly mediating the phosphorylation of Smads or indirectly through activation/ inactivation of required nuclear co-activators that mediate Smad DNA binding. When we treated osteoblasts with the Erk inhibitor, PD98059, it inhibited TGF-β1-induced CTGF promoter activity and protein expression but had no effect on Smad activation or Smad nuclear translocation. Using electro-mobility shift assays we found that treatment with PD98059 impaired transcriptional complex formation on the Smad binding element (SBE) of the CTGF promoter, demonstrating that Erk activation was required for SBE transactivation. Taken together these data demonstrates that Src is an essential upstream signaling partner of both Erk and Smads for TGF-β1 induction of CTGF in osteoblasts, and that Src and Erk have independent effects on Smad signaling required for the formation of a transcriptionally active complex that regulates CTGF promoter activity and expression. Future studies will focus on examining

whether these interactions are unique to osteoblasts compared to other non-osteoblast cells or lines. Evaluation of the precise regulation of CTGF expression in osteoblasts is important to more fully understand its effects on osteoblast differentiation and function.

CROSS-TALK BETWEEN CTGF AND TGF-B1 IN MESENCHYMAL STEM CELL CONDENSATION

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Condensation or the aggregation of mesenchymal stem cells (MSCs) precedes chondrocyte differentiation and is required for cartilage formation. CTGF is a matricellular protein that has been found to be expressed during MSC condensation *in vivo*. It has been reported that CTGF has the ability to bind TGF-β1 and modulate its effects. Using C3H10T1/2 MSCs as a model for mesenchymal condensation, we have shown previously that TGF-β1 induces MSC condensation and this induction is mediated by CTGF. In this study, we were interested in examining whether CTGF treatment or overexpression can mediate MSC condensation in the absence or presence of TGF-β1. C3H10T1/2 MSCs were either treated with rCTGF or infected with adenovirus over-expressing CTGF tagged with GFP. Primary limb bud MSCs treated with rCTGF did not condense. C3H10T1/2 MSCs infected with adenovirus expressing only CTGF demonstrated a 6-7 fold increase in CTGF expression when compared to GFP infected, control cells. Similar to the primary limb experiment, C3H10T1/2 cells infected with CTGF adenovirus did not condense. Surprisingly, TGF-β1 induced MSC condensation was inhibited in cells overexpressing CTGF. These results suggest that sustained overexpression of CTGF is not sufficient to induce mesenchymal cell condensation and has an inhibitory effect on TGF-β1-induced MSC condensation. We next examined the effect of CTGF overexpression on MSC adhesion and spreading associated with vinculin localization at focal adhesions and actin cytoskeletal reorganization. Cells overexpressing CTGF spread more robustly with increased vinculin at sites of focal adhesions associated with the formation of lamelopodia when compared to cells infected with GFP virus alone. We next examined the signaling pathways associated with MAP kinase family and Smads to evaluate differences between TGF-β1-induced MSC condensation and the inhibitory effect of CTGF overexpression on MSC condensation induced by TGF-β1. Cells infected with GFP (control) and CTGF viruses, were treated with TGF-β1 at different time points and examined for the activation of MAP kinases and Smads. Phosphorylated P38, Jnk and Erk were increased in the GFP-infected and CTGF-infected MSCs treated with TGF-β1, suggesting that the MAP kinase family members are not responsible for the inhibitory effect of CTGF over-expression on TGF-β1-induced MSCs condensation. Activation of Smad signaling in CTGF-infected cells treated with TGF-β1 compared to GFP-infected cells treated with TGF-β1, showed a maximum Smad2/3 activation at early time points in cells overexpressing CTGF, compared to GFP-infected cells, suggesting that the Smads signaling pathways might be responsible for the inhibitory effect of CTGF-overexpression on MSCs condensation induced by TGF-β1. Collectively, these data suggest that CTGF by itself does not induce MSCs condensation and that CTGF over-expression inhibits TGF-β1-induced MSCs condensation, at least in part, via changes in Smad activation. Work is underway to examine the role of CTGF ablation during mesenchymal condensation in vivo and determine whether CTGF is sufficient to induce MSCs condensation.

TGF-b RECEPTOR I KINASE ACTIVITY IS REQUIRED FOR CTGF/CCN-2 EXPRESSION INDUCED BY TGF-b AND LPA

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Connective tissue growth factor (CTGF/CCN-2) is the main growth factor that induces fibrosis. CTGF is increased in fibrotic diseases such as cardiac remodeling, renal disorders and muscular dystrophies such as Duchenne. In these diseases and other fibrotic disorders, several extracellular signals have been proposed to regulate the levels of CTGF, and induce the beginning and progression of fibrosis, such as transforming growth factor beta (TGF-b) and lysophosphatidic acid (LPA). In myoblasts we have demonstrated that the expression of CTGF is induced by TGF-b and LPA.

In this study, the regulation of CTGF expression by TGF-b and LPA in skeletal muscle cells was evaluated. TGF-b and LPA increased CTGF expression determined by Northern blot and a specific plasmid reporter for CTGF transcriptional activity containing 5,1 Kb sequence of mouse CTGF promoter. When TGF-b and LPA were added together, CTGF expression augmented in an additive fashion. Interestingly, cells incubated with a specific inhibitor of TGF-b receptor I kinase activity (SB 431542), showed that CTGF expression induced by TGF-b and/or LPA was abrogated. Transfection experiments using plasmids of a dominant negative form of TGF-b receptor II or Smad-7 (both inhibitors of TGF-b signaling pathway) with a plasmid reporter for CTGF transcriptional activity showed that TGF-b pathway was required for CTGF expression induced by LPA and TGF-b. These results suggest a possible crosstalk between TGF-b and LPA-dependent pathway signaling related to CTGF expression. Interestingly, LPA was not able to activate TGF-b pathway signaling by itself evaluated by Smad-2 phosphorylation and Smad-4 nuclear translocation.

This study suggest that TGF-b receptor I kinase activity and Smad dependent pathway are required for CTGF expression induced by TGF-b and particularly by LPA.

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NUCLEAR LOCALIZATION OF CCN5, A CLASSIC MATRICELLULAR PROTEIN

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Hyperproliferation of vascular smooth muscle cells is the hallmark of restenosis following vascular surgery. CCN5, a growth arrest specific protein in smooth muscle cells, dose-dependently inhibits vascular smooth muscle cell proliferation and migration *in vitro.* In the mouse carotid ligation model for vascular injury, CCN5 is lost during the proliferative stages of the response-to-injury process, and returns once the smooth muscle cells stop proliferating and the lesion is mature. Importantly, restoring CCN5 to the carotid artery following the injury process almost completely suppresses smooth muscle cell proliferation and restenosis. Furthermore, CCN5 inhibits proliferation of other smooth muscle cells, including uterine and airway. CCN5, like the other CCN family proteins, was originally discovered as a matricellular protein, a

secreted protein that tightly binds the cell surface. However, recent immunohistochemical and immunofluorescence data in vascular, uterine, and airway smooth muscle cells suggests that CCN5 is also a nuclear protein. Confocal microscopy analysis revealed that CCN5 has a punctate nuclear matrix localization pattern, with complete nucleolar exclusion, in addition to its classical matricellular localization. Therefore, we carried out cell fractionation studies to analyze the subcellular localization of CCN5. Subcellular fractions were separated and analyzed by Western blot analysis with a CCN5 specific antibody. We obtained fractions from two different cell populations: exponentially growing and growth arrested cells. Protein analysis indicates that distinct CCN5 variants are present in both quiescent and exponentially growing cells. However, there are significant differences in which variants are expressed based on subcellular fraction and growth state. Future work aims to characterize the biochemical and functional properties of both nuclear and non-nuclear variants present in smooth muscle cells in different growth states.

EARLY EMBRYONIC EXPRESSION OF CCN5 IN MOUSE EMBRYOS

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To date, CCN5 distribution in early developing rodents has not been mapped comprehensively. CCN5 strongly inhibits adult smooth muscle cell proliferation and motility. Its anti-proliferative action predicts that CCN5 would not be present in developing tissues until the proliferation phase of tissue morphogenesis is complete. However, estrogen induces CCN5 expression in epithelial and smooth muscle cells, suggesting that CCN5 might be widely expressed in embryonic tissues exposed to high levels of estrogen. E9.5–E16.5 dpc murine embryos have already been analyzed by immunohistochemistry. CCN5 was detected in nearly all developing tissues at age E9.5, and gradually developed a more tissue-specific expression pattern as age progressed. The widespread expression pattern of CCN5 in most embryonic tissues suggests a diverse range of functions for CCN5. The importance of CCN5 in early embryonic expression became evident while we were attempting to produce CCN5 overexpressing mice. Our CCN5 construct has been injected several times in order to produce transgenic mice, however litters have not yielded any founder mice, and have been small in size, suggesting a possible embryonic lethality. Current immunohistochemistry studies are focused on CCN5 expression from the fertilized ovum stage until E8.5. These studies have already shown widespread expression of CCN5 as early as E4.5 and all the way up to E8.5. Different IHC techniques are required for embryos younger than E4.5, but these studies are underway. Preliminary Data shows that CCN5 is present in single cell embryos. This data will be not only be important for understanding the function of CCN5, but also critical in the production of our CCN5 overexpressing mouse.

A THREE DIMENSIONAL CELL CULTURE MODEL TO STUDY THE FUNCTION OF CCN6 IN BREAST TUMORIGENESIS

Anupama Pal and Celina G. Kleer

Department of Pathology and Comprehensive Cancer Center, University of Michigan

We have identified a role for CCN6 in breast tumorigenesis and in the regulation of epithelial cell differentiation. We hypothesize that CCN6 mediates epithelial-stromal cross-talk during breast cancer development, and that inhibition of CCN6 in the mammary epithelium may result in morphologic and functional changes toward and invasive phenotype. To test this hypothesis it is necessary to closely recapitulate the microenvironment that the cells encounter *in vivo*. Thus, we set out to develop three dimensional cell cultures with epithelial and stromal components. The stable CCN6-dificient HME cell line was constructed using short hairpin RNA in a lentiviral vector (pLKO.1). shRNA and control cells were cultured in matrigel over a 20 day period and analyzed for their ability to form acini with central lumens. Invasive and metastatic breast cancer cells SUM149 and MDA-MB-231 cells were also cultured under these conditions, and served as controls. Immunofluorescence was performed to detect CCN6, and proteins involved in differentiation including cytokeratins, the myoepithelial cell marker p63, and integrins. We successfully developed 3-dimensional cell cultures of HME shRNA CCN6 and controls, as well as cultures of SUM149 and MDA-MB-231 breast cancer cells. The HME cells transfected with the empty vector control formed well-organized acini with central lumens. We observed that CCN6 inhibition caused a striking morphological change. HME shRNA CCN6 cells did not form acini, but elongated tubular structures with extensive branching and connections, which recapitulated the growth of cancerous cells. These cells also had abnormal localization of integrin staining as well loss of expression of epithelial cell markers. Our results show that three dimensional cultures more closely recapitulates the in vivo setting and allow detailed study of CCN6 function. Given the phenotype that we found, we are exploring the effect of CCN6 on the expression of proteins needed for invasion and degradation of the extracellular medium including matrix metalloproteinases, which will provide insights into CCN6 function in the breast.

INVOLVEMENT OF CYR61 IN GROWTH, MIGRATION, AND METASTASIS OF PROSTATE CANCER CELLS

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Cyr61 has been reported to participate in the development and progression of various cancers, however, its role in prostate caner still remains poorly understood. In the present work, we explored the function of Cyr61 in a series of malignant prostate cancer cell lines, including LnCap, Du145 and PC3. MTT and crystal violet assays demonstrated that Cyr61 was essential to the proliferation of prostate cancer cells. Soft agar assay and xenograft analysis showed that downregulation of Cyr61 suppressed the tumorigenicity of Du145 cells both in vitro and in vivo. Either silencing the cellular Cyr61 by RNAi or neutralizing the endogenous Cyr61 by antibody inhibited the migration of Du145 cells. In contrast, purified protein of Cyr61 promoted the migration of LnCap cells in a does depended manner. These results suggested that Cyr61 was involved in the migration of prostate cancer cells. We also observed the accumulation of mature focal adhesion complexes associated with the impaired migration via Cyr61 downregulation. And further studies showed that Cyr61 regulated the level of activated Rac1 as well as its downstream targets, including phosphorylated JNK, E-Cadherin and p27kip1, which are key molecules involved in cell growth, migration and invasion (Bremnes et al., 2002; Ziober et al., 2001). The in vivo mouse tail vein injection experiment revealed that Cyr61 affected the metastatic capacity of Du145 cells, suggesting that Cyr61 was required for prostate tumor metastasis. Altogether, our results demonstrated that Cyr61 played an important role in the tumorigenicity and metastasis of prostate cancer cells, which will benefit the development of therapeutic strategy for prostate cancers.

URINARY CCN2 AND CCL-2 (MCP-1) ARE PREDICTORS OF INITIATION AND PROGRESSION OF DIABETIC NEPHROPATHY

JoAnn Rambow[1], Fredrick Tam[2], Charles Pusey[2], Melisa Riser[1], and Bruce Riser[1,3]

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[2]Hammersmith Hospital, Imperial College London, UK2, Renal Division, Baxter Health Care, McGaw Park, Il, USA3

Thirty to 40% of diabetic patients will develop diabetic nephropathy, whereas the rest will remain free of this complication for decades. Hypertension and hyperglycemia are established casual factors driving progression once started. However, the initial determinants and the exact pathways remain a mystery. While this is not considered to be an "inflammatory disease" per se, current evidence points to elements of an autoimmune response with possible persistent low levels of inflammation as an initiating factor and a driver of fibrotic progression. We explored cytokines as early factors that may predict those patients destined for diabetic nephropathy, thus allowing an earlier and more focused treatment. CCN2 mRNA is greatly upregulated early in experimental diabetes and is excreted in urine as early as 2 weeks after the onset of hyperglycemia. Another cytokine, macrophage chemoattractant protein-1 (MCP-1 or CCL-2) is often upregulated at sites of inflammation, and is a possible player in diabetic nephropathy. We studied patients assigned to groups, 1) diabetic (D), but without albuminuria (no signs of clinical nephropathy), 2) diabetic with microalbuminuria (DM) (early clinical nephropathy), or 3) diabetic with proteinuria (DP) (advanced nephropathy). Patients were studied over 6 years and were classified as demonstrating progression to renal impairment if serum creatinine rose at least twice, or they became dialysis dependent. Urinary CCL-2/creatinine ratios were significantly higher in patients with DP (3.3-fold higher than D, and 2.1-fold higher than DM patients, $p<0.01$). In contrast, urinary CCN2/creatinine ratios were greatly elevated in both DM and DP patients (125–and 74-fold higher than D patients respectively, $p<0.01$ and $p<0.05$ respectively). Urinary CCL-2 levels, but not CCN2, were also 4-fold higher in patients with diabetic retinopathy as compared to those without retinopathy ($p<0.0005$). Urinary CCL-2, but not CCN2, correlated with the rate of deterioration of estimated glomerular filtration rate (eGFR), $p<0.0005$). In contrast, urinary CCN2, but not CCL-2, correlated with progression of microalbuminuria (, $p<0.05$) and the baseline eGFR (. $p<0.05$). The occurrence of increased urinary CCN2 associated with early disease and CCL-2 with later disease, appears in contrast with the more general paradigm of inflammation followed by fibrosis. It also suggests both that a combination of such new markers may be useful in accessing the risk of progression, and that these cytokines may be important in the initiation and progression of the disease.

This study was supported by project grant from Hammersmith Hospital Special Trustee, the American Diabetes Association (BLR) and the Juvenile Diabetes Association (BLR).

CCN3/NOV IS RECRUITED, TRAFFICKED AND MODIFIED DURING ALL-TRANS RETINOIC ACID INDUCED NEURONAL DIFFERENTIATION ON A 3D MATRIX: ANALYSIS USING DOMAIN SPECIFIC ANTIBODIES

Micky Tsui[1], Nissi Wei[1], Bernard Perbal[2], Herman Yeger[1]

[1]Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Canada [2] Laboratoire d' Oncologie Virale et Moléculaire, Université paris7, UFR de Biochimie, Paris, France

CCN3/NOV is a secretable, matrix associated protein that has previously been demonstrated to be expressed in different areas of the early developing brain of rats and has been postulated to play a role in the establishment and maintenance of specific brain functions. We developed an in-vitro CNS like neuronal differentiation model using the NT2/D1 teratocarcinoma cell line grown on a 3D matrix to assay the role of CCN3 during neuronaldifferentiation.

The differentiation of neurons was accomplished by treating NT2/D1 with all-trans retinoic acid (ATRA) over 15 days on an Ultraweb matrix that has been shown to simulate 3D in-vivo matrix morphology. Neuronal maturation was stimulated by cell cycle inhibitors cytosine arabinoside, uridine and 5-fluoro-2'-deoxyuridine for an additional 5 days. CCN3 expression was assessed at 5 day intervals by western blot and immunocytochemistry using domain specific CCN3 antibodies (NH2-NH5) Following ATRA induced differentiation, detection of full-length 46 KDa CCN3 decreased with the NH2 antibody and dramatically increased with NH4. Detection of the truncated 32 KDa CCN3 form by the NH3 and NH4 antibodies was also dramatically increased. While there was little change detected in the 46 KDa CCN3 using the original K19M antibody, a larger 48 KDa band, suggesting post translational modification, was detected following the appearance of mature looking neurons. Secretion of the 46 KDa form of CCN3 into the medium was detected starting from day 10 coincident with increased neurofilament expression. Immunocytochemistry showed an increase in cytoplasmic expression and also nuclear localization of CCN3 in neurites with K19M, NH3, NH4 and NH5. K19M, NH4 and NH5 antibodies were also able to localize CCN3 in neuronal processes.

We conclude that CCN3 likely plays a key role during ATRA induced neuronal differentiation, as seen by its increased nuclear trafficking and expression. Increased secretion by maturing neurons suggests that CCN3 could have an extracellular role during neuronal differentiation. Differential detection of CCN3 by antibodies directed against the different domains of CCN3 suggests possible functional relevant conformational changes and protein interactions during neuronal differentiation and maturation.

PROGRAM

Saturday October 18, 2008

20:00
Herman Yeger
Welcome

Bernard Perbal
Opening Remarks

20:30
Peter Butler, Bernard Perbal
Springer Award Presentation

21:00
Paul Bornstein
Plenary Lecture
Matricellular proteins regulate cell function: studies of thrombospondins 1 and 2

Sunday October 19, 2008

Session I: CCN Structure/Function; Expression (I)
Chairs: Gary Fisher; Masaharu Takigawa

8:30–8:55
Ravi Acharya
The CCN family of proteins: structure-function relationships

8:55–9:20
Satoshi Kubota
Nucleophosmin/b23: a multifunctional regulator that determines the fate of CCN2 mRNA

9:20–9:45
Toshihiro Ohgawara
Regulation of chondrocytic phenotype by microRNA 18a: involvement of CCN2/CTGF as a major target gene

9:45–10:10
Claudio Cabello-Verrugio
CTGF/CCN2 expression induced by TGF-b is modulated through a mechanism dependent of decorin and LRP-1

10:10–10:35
Chundo Oh
identification of sox9 binding site in ccn2 (CTGF) gene by use of CHip on CHIP analysis

Session II: CCN Structure/Function; Expression (II)
Chairs: John Castellot; **Enrique Brandan**

11:00–11:25
Brahim Chaqour
Mechanical regulation of the Cyr61/CCN1 gene Requires the combined activity of the myocardin-related transcription factor (MRTF) -A and P300/CBP in smooth muscle cells

11:25–11:50
Douglas Hamilton
Periostin is Expressed in Human Skin and is Regulated by Mechanical Strain

11:50–12:15
Lan Wei
Functional and biochemical assessment of CCN5 targeted to the nucleus

12:15–12:40
Hirokazu Okada
Poly(ADP-ribose) polymerase-1 (parp-1) and parp-1 binding element (pbe) enhance murine ccn2 gene transcription in proximal tubular epithelial cells (ptec)

Session III: Osteogenesis and Chondrogenesis (I)
Chairs: David Brigstock; Robert Lafyatis

14:15–14:40
Masaharu Takigawa
Roles of CCN2 in skeletal growth and regeneration–requirement for both endochondral and intramembranous bone formation-

14:40–15:05
Fayez Safadi
Skeletal phenotype in transgenic mice over-expressing CTGF in cells of the osteoblast lineage

15:05–15:30
Faith Hall-Glenn
CCN1 and CCN2 are essential for chondrogenesis

15:30–15:55
Ken-ichi Katsube
CCN3 and NOTCH/BMP signals

15:55–16:20
Andrew Leask
FAK/SRC suppresses early chondrogenesis: central role of CCN2

Session IV: Osteogenesis and Chondrogenesis (II)
Chairs: Havard Attramadal; Ben Alman

16:40–17:05
Mark Erwin
CTGF/CCN-2 is produced by non-chondrodystrophic canine intervertebral disc-derived notochordal cells and upregulate nucleus pulposus aggrecan gene expression

17:05–17:30
Karen Lyons
CCN2 is required for vascular remodeling in vivo

17:30–17:55
Mukesh Jain
KLF15 regulates the cardiac response to stress

17:55–18:20
Ursula Kees
The role of CTGF in paediatric acute lymphoblastic leukaemia

Monday October 20, 2008

Session V: Pathobiology (I)
Chairs: Ken-Ichy Katsube; Satoshi Kubota

8:30–8:55
Laure Rittié
Expression of CCN proteins in normal human skin in vivo and after wound healing

8:55–9:20
Andrew Leask
Connective tissue growth factor promoter activity in normal and wounded skin

9:20–9:45
Taihao Quan
Ultraviolet irradiation induces CYR61/CCN1, a novel mediator of collagen homeostasis, via activation of transcription factor AP-1 in human skin dermal fibroblasts

9:45–10:10
Kirsten Bielefeld
The interaction of β-catenin with extra-cellular matrix components in dermal fibroblasts during wound healing

10:10–10:35
George Yang
The role of the matricellular protein del1 in bone fracture healing

Session VI: Pathobiology (II)
Chairs: Lester Lau; Margarete Goppelt-Struebe

11:00–11:25
Mary Lou Cutler
Connective tissue growth factor (CTGF/CCN2) as a regulator of β1 integrin-mediated cell adhesion and survival during lactogenic differentiation of mouse mammary epithelial cells

11:25–11:50
M. Shakil Ahmed
CCN2/CTGF–cardioprotective factor in myocardial ischemia/reperfusion injury and heart failure

11:50–12:15
Stephen Twigg
High glucose and free fatty acid adverse effects on cardiac myocytes are mediated by ccn2/connective tissue growth factor (CTGF) through TrkA

12:15–12:40
Roel Goldschmeding
Balancing CCN-2 (CTGF) and BMP(s) in complications of diabetes mellitus

Session VII: Fibrosis (I)
Chairs: Brahim Chaqour; Bruce Riser

14:10–14:35
Takako Hattori
CCN2/CTGF is transactivated through its enhancer element by Sox9 in fibroblasts: possible roles in fibrosis

14:35–15:00
Joshua Russo
In vivo effects of CCN5 overexpression in fibroids

15:00–15:25
Shiwen Xu
Microvascular pericytes express CCN2 in tissue fibrosis

15:25–15:50
Alpdogan Kantarci
Epithelial and connective tissue cell CTGF/CCN2 expression in gingival fibrosis: role of epithelial-mesenchymal transition

Session VIII: Fibrosis (II)
Chairs: Karen Lyons; Andrew Leask]

16:20–16:45
Enrique Brandan
Fibrosis in duchenne muscular dystrophy; role of CCN2 and its potential inhibition by decorin

16:45–17:10
Philip Trackman
Tissue-specific regulation of CCN2/CTGF in gingival fibroblasts and its relationship to gingival fibrosis

17:10–17:35
Bruce Riser
CCN3 (Nov) is a negative regulator of CCN2 (CTGF) and a novel endogenous inhibitor of fibrosis in experimental nephropathy

17:35–18:00
David R Brigstock
Targeted CCN2 gene therapy in experimental liver fibrosis

18:00–18:25
Margarete Goppelt-Struebe
Cell type-specific regulation of connective tissue growth factor by hypoxia

Tuesday October 21, 2008

Educational Session: From Matricellular to Extracellular
Chairs: Paul Bornstein, David Brigstock, Margarete Goppelt-Struebe, Lester Lau, Karen Lyons, Andrew Leask, Bernard Perbal, Masaharu Takigawa

9:00–9:35
Aleksander Hinek
Elastin receptor subunits facilitate elastic fiber assembly and modulate cell proliferation

9:35–10:10
William G. Cole
Abnormal extracellular matrices in rare and common disorders

10:40–11:15
Ren-Ke Li,
Cell transplantation and gene therapy preserves matrix homeostasis: a novel paracrine mechanism

11:15–11:50
Katharine Sodek,
MT1-MMP and contractility mediate invasive behavior by ovarian cancer cells

Round Table Discussion: 11:50–12:10

Session IX: Pathobiology (III)
Chairs: Celina Kleer; Ruth Lupu

13:45–14:10
Viviana Vallacchi
CCN3 promotes melanoma progression by regulating integrin expression, adhesion and chemoresistance

14:10–14:35
Lynn McCallum
CCN3 restores growth regulation in Chronic Myeloid Leukaemia

14:35–15:00
Katia Scotlandi
Prognostic role of CCN3 in ostesarcoma and Ewing's sarcoma

15:00–15:25
Adam A. Sabile
CCN1/cyr61 enhances the metastatic potential of human osteosarcoma cell lines and activates the AKT pathway

Session X: Pathobiology (IV)

Chairs: Philip Trackman

16:00–16:25
Wun-Chey Sin
CCN3 suppresses growth and induces actin cytoskeletal reorganization in breast cancer cells

16:25–16:50
Ruth Lupu
CCN1: A new target for chemotherapeutical drugs in breast cancer treatment

16:50–17:15
Sushanta Banerjee
Suppression of invasive front by CCN5/wisp-2 in breast cancer is mediated through silencing of microRNA-10b

17:15–17:40
Yves Courty
Identification of CCN proteins as substrates for kallikrein-related peptidases

Wednesday October 22, 2008

Springer Scholarships
Chairs: Stephen Twigg; Sandra Irvine

8:30–8:55
Wei Huang
The CCN family member CCN6: Inhibition of CCN6 regulates E-cadherin expression in the breast epithelium through up-regulation of Snail and Zeb1

8:55–9:20
Ingrid Espinoza
The CCN1/α6β1 connection and the hormonal response in breast cancer

9:20–9:45
Takanori Eguchi
Novel transcriptional regulation of CCN2/CTGF by nuclear translocated MMP3

CCN in Cell Death and Survival

9:50–10:15
Lester Lau
Integrin-mediated matrix signaling in cell death and survival

10:15–11:15
Concluding remarks and presentation of the next meeting