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ABSTRACTS

Seventh international workshop on the CCN family of genes

October 16–19, 2013, NICE, France

Organizers:
Annick and Bernard Perbal

Scientific Advisory Board:
D. Brigstock, L. Lau, A. Leask, M. Takigawa

PROGRAM OF THE 7TH INTERNATIONAL WORKSHOP ON THE CCN FAMILY OF GENES

Wednesday, October 16, 2013

17h00 Registration at the MERCURE Hotel begins

19h00 **Welcome Reception and Buffet Dinner**

20h30
Bernard Perbal
Introductory conference
CCN proteins: A Centralized Communication Network

Thursday, October 17, 2013

Session I
PATHOBIOLOGY OF CCN PROTEINS (I) - Cancer
Chairpersons: Bernard Perbal and Enrique Brandan

8:45
Bernard Perbal
Introduction

9h00–9h25
Sushanta Banerjee
A CCN5 balancing act on "grow and go" of p27 tumor suppressor protein in breast cancer cells

9h25–9h50
Celina Kleer
Novel roles of CCN6 in mesenchymal-epithelial transition (MET) and loss of stem cell properties of breast cancer cells

9h50–10h15
Ursula Kees
Pathophysiology of CTGF/CCN2 expression in acute lymphoblastic leukaemia

10h15–10h40 **Coffee Break**

10h40–11h10
Ruth Lupu
Direct involvement of CCN1 in breast cancer acquisition of antiestrogen resistance

11h10–11h35
Adam Sabile
Caprin-1, a novel cyr61/CCN1-interacting protein, promotes osteosarcoma tumor growth and lung metastasis in mice

11h35–12h00
Georgia Schäfer
Tumour cells down-regulate CCN2 gene expression in co-cultured fibroblasts in a smad7- and erk-dependent manner

12h00–14h00 **Lunch**

ICCNS-SPRINGER AWARD SESSION

14h00 *Award Presentation - Bernard Perbal*

14h10–15h10
Carlo Croce
Causes and consequences of microRNA dysregulation in cancer

15h30–16h00 **Break**

Session II
REGULATION OF CCN PROTEINS FUNCTIONS BY MicroRNAS
Chairperson: Carlo Croce

16h00–16h25
Sandra Irvine
Inhibiting mir-130a/b restores ccn3, induces apoptosis and cell cycle regulation in chronic myeloid leukaemia cells

16h25–16h50
Chaya Brodie
Mir–145 regulates the migration and stemness of glioma stem cells by targeting CTGF/CCN2: molecular mechanisms and signaling pathways.

16h50–17h15
David Brigstock
Regulation of CCN2 fibrogenic pathways by exosomal microRNA

19h00 **ICCNS-Sponsored Social Event**

Friday, October 18, 2013

Session III
PATHOBIOLOGY OF CCN PROTEINS - II – Metabolism and differentiation
Chairpersons: Philip Trackman and Havard Attramadal

8h20
Philip Trackman
Introduction

8h35–9h00
Margarete Goppelt-Struebe
Cell type-specific regulation of CCN2 expression in the kidney

9h00–9h25
Jean Buteau
Potential role of CCN3 in type 2 diabetes

9h25–9h50
Ulf Smith
WISP2/CCN5 as a regulator of PPARγ in mesenchymal cells

9h50–10h20 **Coffee Break**

10h20–10h45
John Castellot
CCN5 in uterine fibroids and oxygen injury in lung: a paradox in function and mechanism

10h45–11h10
Mitsuaki Ono
CCN4/WISP-1 enhances cell migration during skin wound healing

11h10–11h35
Muriel Cario-André
Are CCN family molecules implicated in the regulation of pigmentation? The example of systemic sclerosis

11h35–12h00
Brahim Chaqour
Role of CCN2 in developmental and pathological angiogenesis in the retina

12h00–13h45 **Lunch**

Session IV
PATHOBIOLOGY OF CCN PROTEINS - III - Fibrosis
Chairs: Roel Goldschmeding and John Castellot

13h45
John Castellot
Introduction

14h00–14h25
Lester Lau
CCN1 functions in liver fibrosis and cancer

14h25–14h50
Andrew Leask
Skin progenitor cells contribute to bleomycin-induced skin fibrosis

14h50–15h15
Roel Goldschmeding
CCN2 as a target for antifibrotic therapy in kidney disease

15h15–15:40
Mary Barbe
Long-term performance of a high demand repetitive tasks induces both increased serum and muscle CTGF/CCN2 and motor declines.

15h40–16h10 **Break**

16h10–16h35
Enrique Brandan
Novel mechanisms involved in the development of fibrosis associated to Duchenne muscular dystrophy

16h35–17h00
Joanna Nikitorowicz-Buniak
Abnormally differentiating keratinocytes in systemic sclerosis epidermis show enhanced secretion of CCN2

17h00–17h25
Richard Stratton
Investigating the role of MRTF-A in systemic sclerosis

17h25 **SHORT ORAL PRESENTATIONS OF POSTERS**

Evening on your own

Saturday, October 19, 2013

Session V
CCN BIOLOGY: FROM GENES TO PROTEINS
Chairpersons: Masaharu Takigawa and David Brigstock

8h45
David Brigstock
Introduction

9h00–9h25
Philip Trackman
Mechanisms of increased gingival collagen accumulation by CCN2

9h25–9h50
Fabien Gueugnon
CCN expression and fragmentation in the lungs

9h50–10h15
Satoshi Kubota
New functional aspects of known molecules as CCN2 partners

10h15–10h45 **Coffee Break**

10h45–11h10
Fan-E Mo
Matricellular protein CCN1 regulates cardiomyocyte apoptosis in mice with stress-induced cardiac injury

11h10–11h40
Havard Attramadal
Implications of CCN2 in infarct healing and myocardial remodeling after myocardial infarction11h45–13h00 **Lunch**

Session VI
BIOLOGICAL FUNCTIONS OF CCN PROTEINS IN DEVELOPMENT
Chairpersons: Lester Lau and Andrew Leask

13h00
Lester Lau
Introduction

13h15–13h40
Masaharu Takigawa
Roles of CCN2 and CCN3 in skeletogenesis

13h40–14h15
Takako Hattori
Cartilage-specific overexpression of CCN3 modulates endochondral bone formation

14h15–14h40
Tarek Abd El Kader
Evaluating the regenerative effect of CCN2 independent modules on chondrocytes in vitro and osteoarthritis models in vivo

14h40–15h05
Akira Yamaguchi
Role of CCN3 in osteoblast differentiation and bone regeneration

15h05–15h25 **Break**

SPRINGER SCHOLARSHIPS AWARDS

15h25–15h50
Weike Si
CCN1 is a target gene of β-catenin signaling and play an important role in the progression of liver cirrhotic and hepatocellular carcinoma

15h50–16h15
Meegan Howlett
High levels of connective tissue growth factor/CCN2 can accelerate disease in a model of acute lymphoblastic leukaemia

16h15–16h40
Alyssa Charrier
CCN2 is present in exosomes from activated hepatic stellate cells

16h40
Bernard Perbal
Concluding remarks

19h30 **COCKTAIL AND GALA DINNER WITH LIVE MUSIC**

Sunday, October 20, 2013

Optional Tour - Visit of Monaco

Departure of Participants

ABSTRACTS

PATHOBIOLOGY OF CCN PROTEINS

PART I: CANCER

A CCN5 BALANCING ACT ON "GROWAND GO" OF P27 TUMOR SUPPRESSOR PROTEIN IN BREAST CANCER CELLS

Inamul Haque, Archana De, Gargi Maity, Sandipto Sarkar, Snigdh Banerjee, Sushanta K. Banerjee

Division of Hematology and Oncology and
Department of Anatomy and Cell Biology
University of Kansas Medical Center, Kansas City, KS

Breast cancer progression is a complex and multistep process. Findings from our laboratory and others demonstrate that a matricellular protein CCN5, which is also known as*WISP-2*, participates negatively in this complex process. A role of CCN5 in prevention of tumor progression was first suggested in pancreatic cancer. Subsequently, an increasing number of studies on breast cancer cells established an anti-invasive role of CCN5 in breast cancer progression as it participates in precluding cancer cell growth, morphologic alterations through the prevention of epithelial-to-mesenchymal transition (EMT) and invasion of breast cancer cells through the regulation of microRNA-10b. Intrinsic loss or deficiency of CCN5 in breast cancer cells, which makes cancer cells more aggressive, could be mediated through subsequent genetic lesions such as mutational activation of p53 tumor suppressor genes. However, these mechanisms still do not explain precisely how CCN5 affects breast tumor cell growth and progression. Present studies reveal that CCN5 regulates the tumor cells growth and their progression through regulation of $p27^{Kip1}$ protein. It enhances $p27^{Kip1}$ accumulation in the nucleus as well as prevents nuclear-to-cytoplasmic mislocalization or abnormal accumulation of $p27^{Kip1}$ in the cytoplasm, which is liable for carcinogenic growth and aggressive behavior of breast cancer cells. Collectively, our results provide support for the clinical development of CCN5 agonists for the treatment of breast and pancreatic cancer.

NOVEL ROLES OF CCN6 IN MESENCHYMAL-EPITHELIAL TRANSITION (MET) AND LOSS OF STEM CELL PROPERTIES OF BREAST CANCER CELLS

Wei Huang, Ph.D., Kathy Toy, B.S., and Celina G. Kleer

University of Michigan Department of Pathology and Comprehensive Cancer Center, Ann Arbor, Michigan, USA
Background: Epithelial-mesenchymal transition (EMT) and its reverse process, mesenchymal-epithelial transition (MET), play key roles in regulating embryogenesis. Recent studies have shown that aberrant EMT activation contributes to cancer progression and metastasis. Links between EMT and the gain of stem cell properties have been suggested in physiological and pathological situations. CCN family protein 6 (CCN6/WISP3) is a novel tumor suppressor that regulates diverse cellular functions. Our previous studies have shown the blockade of CCN6 by short hairpin RNA triggers EMT in benign breast epithelial cells, with decreased expression of epithelial marker E-cadherin and increased activation of corresponding transcriptional repressor, *Zeb1* and *Snail*. Moreover, loss of CCN6 expression endows benign breast epithelial cells with growth factor-independent properties, such as cell survival and anoikis resistance. However, little is known regarding the functions of CCN6 overexpression in breast cancer progression. **Methods:** We overexpressed CCN6 in breast cancer cell lines, MDA-MB-231 and SUM-159, which are aggressive, have mesenchymal-like features and low levels of endogenous CCN6. CCN6 provoked functions were studied *in vitro* by proliferation, motility and invasion assays. Aldehyde dehydrogenase (ALDH1) was used to identify and investigate CCN6 roles in human breast cancer stem cells in vitro and in the mammary fat pads of mice. **Results:** We showed that overexpression of CCN6 inhibits cell proliferation, motility and invasion of breast cancer cells. Importantly, CCN6 overexpression in these cells led to phenotypic changes towards mesenchymal-epithelial transition (MET), characterized by loss of mesenchymal cell marker Vimentin and downregulation of transcriptional factor, *Zeb1*. CCN6 overexpression significantly reduced the stem cell population of MDA-MB-231 and SUM-159 breast cancer cells, as well as their ability to form primary and metastatic tumors in mice. **Conclusions**: Our data suggest a novel function of CCN6 in regulating MET and reducing the stem cell population of aggressive breast cancer cells.

PATHOPHYSIOLOGY OF *CTGF/CCN2* EXPRESSION IN ACUTE LYMPHOBLASTIC LEUKAEMIA

Mathew D Welch[1], Jette Ford[1], Julia E Wells[1], Meegan Howlett[1], David R Brigstock[2] and Ursula R Kees[1]

[1]Telethon Institute for Child Health Research, Centre for Child Health Research, University of Western Australia, Perth, Australia, [2]Center for Clinical and Translational Research, The Research Institute at Nationwide Children's Hospital, Columbus, Ohio, USA

High expression of connective tissue growth factor (*CTGF/CCN2*) is one of the most prevalent genetic changes in B-cell precursor acute lymphoblastic leukaemia (BCP-ALL), present in 75 % of cases. We reported considerable variation in *CTGF* expression across patient subgroups (Boag et al, Br J Haem 2007) and studies in children and adults documented a link between high *CTGF* expression and therapy resistance. To examine the underlying mechanisms we generated a panel of BCP-ALL cell lines grown from patient specimens, and they showed very high, intermediate or non-detectable *CTGF* expression. We investigated whether epigenetic regulation was causing high expression and found that methylation of the *CTGF* CpG island correlated with gene expression (Welch et al, Br J Haem 2013). We concluded that hypomethylation of the locus is recurrent in BCP-ALL, and is required but not sufficient to promote deregulated *CTGF* expression.

The patient-derived cell lines secrete full length 38 kDa CTGF. Since CTGF is normally expressed in bone marrow stromal cells, and malignant BCP-ALL cells secrete CTGF to surrounding cells, we examined the effect of rCTGF on HS-5 and T-MSC bone marrow stromal cells, and recorded significantly increased proliferation. Next we incubated HS-5 cells with rCTGF for 8 h and found 1409 genes significantly deregulated >2-fold compared to control. We identified the profile to reflect a signature reported for tumour-associated fibroblasts, known to be a powerful predictor of clinical course. The significantly up-regulated genes included membrane-bound *ITGAV, ABCA1, GPRC5B*, ECM genes *COL1A2, COL3A1, COL5A1, FN1, LOX* and *LUM*, as well as chemokine *CCL2*. This demonstrates that CTGF changes the gene expression profile in stromal cells, making them capable of modifying the tumour microenvironment.

To determine whether bone marrow stromal cells have the capacity to influence resistance to chemotherapeutic drugs, we measured the effect of four drugs used in the therapy of BCP-ALL patients, comprising vincristine (VCR), cytosine arabinoside (ARAC), dexamethasone (DEX) and L-asparaginase (LASP). In the presence of HS-5 cells, significant protection from VCR and ARAC was achieved (Tesfai et al, Leukemia Research 2012). We used genetically engineered BCP-ALL cells in our NOD/SCID xenograft model and demonstrated that the therapeutic effect of ARAC was abolished in BCP-ALL cells that express *CTGF* at high levels. Taken together we conclude that *CTGF* mediates drug resistance in BCP-ALL. Current studies aim to further delineate the interactions in the microenvironment, with the goal to develop more effective therapy for patients.

DIRECT INVOLVEMENT OF CCN1 IN BREAST CANCER ACQUISITION OF ANTIESTROGEN RESISTANCE

Ingrid Espinoza[1], Anatilde Gonzalez-Guerrico[1], Lin Yang[1], Ruth Lupu[1]

Department Experimental Medicine and Pathology and Biochemistry and Molecular Biology
Mayo Clinic Cancer Center, Mayo Clinic Rochester, MN 55905

The pro-angiogenic factor CCN1 is expressed in about 48 % of invasive breast carcinomas. More than 60 % of human breast cancer tumors express Estrogen Receptor (ER) and many of those are Estrogen (E_2) dependent. ER positive tumors are considered to have a good prognosis; however many of these tumors escape the E_2 requirements and become E_2 independent. We show that CCN1 promotes breast cancer cells and tumors to bypass the E_2 requirements for proliferation and tumorigenesis. In addition, CCN1 induces *de novo* acquisition of antiestrogen resistance *in vitro* (in culture) and *in vivo* (athymic nude mice). The role(s) of CCN1 in hormonal response and ER transcriptional activation in breast cancer is not well understood. Thus, the identification of molecules that regulate ER function may facilitate development of breast cancer treatment strategies. We first demonstrated that CCN1 induces constitutive ER transcriptional activation independent from E_2. We showed that CCN1 internalizes to the nucleus and CCN1 binds and co-localizes with the ER. Additionally, CCN1 transcriptional activity is regulated, at least in part, by Foxo3a. In search of the mechanism by which CCN1 that modulates hormonal response, we discovered that CCN1 and Foxo3a interact in the presence of E_2. The functional interaction between CCN1/ER/Foxo3a proteins, most likely disrupt the ability of cell to respond to hormonal modulators of growth. Furthermore, this studies demonstrate that CYR 61 activate Foxo3a are novel therapeutic targets to halt hormone-independent breast carcinomas.

CAPRIN-1, A NOVEL CYR61/CCN1-INTERACTING PROTEIN, PROMOTES OSTEOSARCOMA TUMOR GROWTH AND LUNG METASTASIS IN MICE

Sabile A[1], Arlt M[1], Muff R[1], Husmann K[1], Hess D[2], Bertz J[1], Langsam B[1], Aemisegger C[3], Ziegler U[3], Born W[1] and Fuchs B[1]

1: University Hospital Balgrist, Department of Orthopedics, Zurich
2: Friedrich Miescher Institute, Basel
3: Center for Microscopy and Image Analysis, University of Zurich

Osteosarcoma (OS) is the most common primary bone malignancy in children and adolescents. More than 30 % of patients develop lung metastasis, which is the leading cause of mortality. Consequently, suitable early diagnostic markers of metastasizing OS and corresponding treatment targets are urgently needed. Recently, the extracellular matrix protein Cyr61 has been recognized as a malignancy promoting protein in OS mouse model with prognostic potential in human OS. In the present study, we aimed at the investigation of the role of Caprin-1, a novel Cyr61/CCN1-interacting protein, in OS pathogenesis and metastasis formation.

We combined endogenous Cyr61/CCN1 immunoprecipitation and mass spectrometric analysis to identify Caprin-1 as a new Cyr61/CCN1-interacting protein. Then, we investigated the subcellular localization of Cyr61/CCN1 and Caprin-1 using a detailed confocal microscopy analysis. Finally, we used an orthotopic OS mouse model to investigate the in vivo effects of Caprin-1 overexpression.

Stable overexpression of Caprin-1 in human OS cells resulted in the formation of Caprin-1 and Cyr61/CCN1 containing stress granules. Furthermore, we showed that Caprin-1 overexpression in OS cell lines enhanced their resistance to cisplatin. Importantly, SCID mice intratibially injected with OS SaOS-2 cells stably overexpressing Caprin-1 showed accelerated primary tumor growth and a remarkably increased lung metastatic load

compared to mice injected with control SaOS-2 cells. Consequently, the survival of mice with Caprin-1 overexpressing tumors was significantly shorter than that of mice injected with control SaOS-2 cells.
In conclusion, overexpression of Caprin-1, a novel Cyr61/CCN1-interacting protein, in the human osteoblastic SaOS-2 OS cell line provoked co-accumulation with Cyr61 in stress granules, which are known to have apoptosis protective functions. This is consistent with the here reported more resistant phenotype of cells overexpressing Caprin-1 to cisplatin. Importantly, OS cells overexpressing Caprin-1 showed strikingly enhanced malignant phenotype compared to control cells in mouse experimental intratibial OS. From this and our previous study, we conclude that both Caprin-1 and Cyr61/CCN1, have related but distinct OS malignancy-enhancing properties in addition to largely unknown interaction-dependent functions, which are currently under investigation in our laboratory.

TUMOUR CELLS DOWN-REGULATE CCN2 GENE EXPRESSION IN CO-CULTURED FIBROBLASTS IN A SMAD7- AND ERK-DEPENDENT MANNER

Beverley A. van Rooyen[1,2], Georgia Schäfer[1,2], Virna D. Leaner[1] and M. Iqbal Parker[1,2]

[1] Division of Medical Biochemistry & the Institute of Infectious Diseases and Molecular Medicine, Faculty of Health Sciences, University of Cape Town, South Africa
[2] International Centre for Genetic Engineering and Biotechnology (ICGEB), Anzio Rd, Observatory, 7925, South Africa

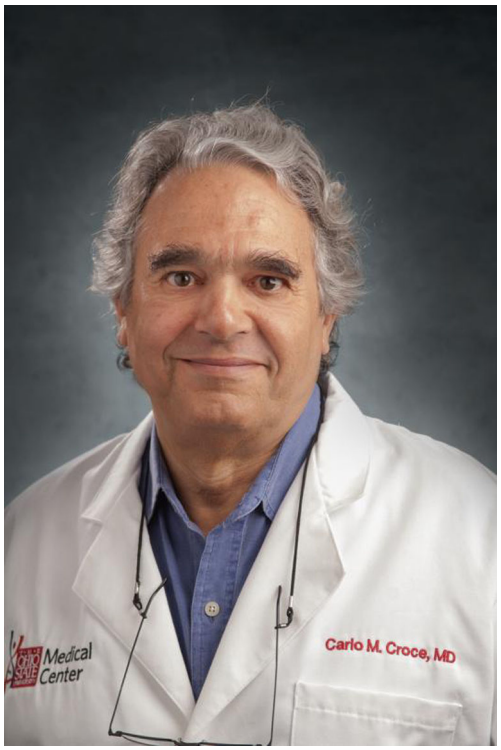
Recent studies have revealed that interactions between tumour cells and the surrounding stroma play an important role in facilitating tumour growth and invasion. The aim of this study was to investigate the mechanisms involved in tumour cell-mediated regulation of extracellular matrix and adhesion molecule production in co-cultured fibroblasts. To this end, microarray analysis was performed on CCD-1068SK human fibroblast cells after direct co-culture with MDA-MB-231 human breast tumour cells. We found that the expression of both connective tissue growth factor (CTGF/CCN2) and type I collagen was negatively regulated in normal CCD-1068SK fibroblasts under direct co-culture conditions. Further analysis revealed that Smad7, a known negative regulator of the Smad signalling pathway involved in CCN2 promoter activity, was increased in directly co-cultured fibroblasts. Inhibition of Smad7 expression in CCD-1068SK fibroblasts resulted in increased CCN2 expression, while Smad7 overexpression had the opposite effect. Silencing CCN2 gene expression in fibroblasts led, in turn, to a decrease in type I collagen mRNA and protein levels. ERK signalling was also impaired in CCD-1068SK fibroblasts after direct co-culture with MDA-MB-231 tumour cells, with Smad7 overexpression in fibroblasts leading to a similar decrease in ERK activity. These effects, however, were not seen in fibroblasts that were indirectly co-cultured with tumour cells. We therefore conclude that breast cancer cells require close contact with fibroblasts in order to upregulate Smad7 which, in turn, leads to decreased ERK signalling resulting in diminished expression of the stromal proteins CCN2 and type I collagen.

ICCNS - SPRINGER AWARD CONFERENCE

Professor Carlo Croce

Professor and Chair of Molecular Virology, Immunology and Medical Genetics,
Director of the Human Cancer Genetics Program,
Director of the Institute of Genetics

The Ohio State University Comprehensive Cancer Center
Columbus, Ohio, USA



CAUSES AND CONSEQUENCES OF MICRO-RNA DYSREGULATION IN CANCER

REGULATION OF CCN PROTEIN FUNCTIONS BY MICRO-RNAS

INHIBITING MIR-130A/B RESTORES CCN3, INDUCES APOPTOSIS AND CELL CYCLE REGULATION IN CHRONIC MYELOID LEUKAEMIA CELLS

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Background: Chronic Myeloid Leukaemia (CML) is characterized by the presence of the BCR-ABL fusion gene. MicroRNAs 130a and 130b

are BCR-ABL dependent and suppress the function of the tumour suppressor, CCN3, in CML. We have investigated the effects of inhibiting miR-130a/b activity in the K562 CML cell line.
Methods: To investigate the inhibitory effects of miR-130a/b activity, antimiR sequences against miR-130a/b were transfected into K562 cells. Colony formation and cell cycle analysis by flow cytometry was assayed. The expression levels of CCN3, CDKIs p16, p21, p27, and the expression of pERK1/2, pAKT, pSTAT5 were quantified.
Results: Significant induction of CCN3 transcripts and protein was observed with miR-130a/b knockdown. K562 clonogenicity decreased >50 % with both miR-130a ($p<0.05$) and miR-130b ($p<0.01$) inhibition. Higher sub-G0 events were observed with miR-130a (8.7) and miR-130b (13.5) compared to the scrambled control (5.5). Significant induction of cleaved PARP and caspase-3 confirmed apoptosis. Both miR-130a and miR-130b knockdown induced p16, p27 and inhibited p21, pERK1/2 and pAKT.
Conclusion: Our results suggest an oncogenic role for miR-130a/b in CML. Inhibition of these miRNAs causes antiproliferative effect in CML by inducing CCN3, inhibiting mitogenic signalling and restoring cell cycle regulation.

MIR-145 REGULATES THE MIGRATION AND STEMNESS OF GLIOMA STEM CELLS BY TARGETING CTGF/CCN2: MOLECULAR MECHANISMS AND SIGNALING PATHWAYS

Chaya Brodie[1,2], Ariel Bier[1], Hae-Kyung Lee[2], Simona Cazacu[2], Susan Finniss[2] and Cunli Xiang[2]

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Glioblastoma (GBM), the most common and aggressive malignant astrocytic tumors, are characterized by increased invasion into the surrounding brain tissues. GBM contain a small subpopulation of cancer stem cells (GSCs) that are implicated in therapeutic resistance, and tumor infiltration and recurrence. In this study we examined the expression and function of miR-145 in glial tumors and GSCs and the molecular mechanisms that mediate miR-145 effects. Using TCGA analysis and real-time PCR we found that the expression of miR-145/143 cluster was downregulated in astrocytic tumors compared to normal brain specimens and in glioma cells and glioma stem cells (GSCs) compared to normal astrocytes and neural stem cells. Moreover, the low expression of both miR-145 in GBM was correlated with poor patient prognosis. Transfection of the cells with a miR-145 mimic or transduction with a lentivirus vector expressing pre-miR 145 significantly decreased the migration and invasion of glioma cells and the self renewal of GSCs. We identified connective tissue growth factor (CTGF) as a novel target of miR-145 in glioma cells; transfection of the cells with this miRNA decreased the expression of CTGF as determined by Western blot analysis and the expression of its 3′-UTR fused to luciferase. Moreover, overexpression of a CTGF plasmid lacking the 3′-UTR and administration of recombinant CTGF protein abrogated the inhibitory effect of miR-145 on GSC migration and the self-renewal. Similarly, silencing of CTGF decreased the migration of glioma cells and the stemness characteristics of GSCs. We further found that CTGF silencing decreased the expression of SPARC, phospho-FAK and FAK and overexpression of SPARC abrogated the inhibitory effect of CTGF silencing on cell migration. In addition, miR-145 and silencing of CTGF decreased the expression of SOX2 and Nanog, which contributed to their inhibitory effect on the stemness of GSCs. These results demonstrate that miR-145 is downregulated in glial tumors and in GSCs and its low expression in GBM predicts poor patient prognosis. In addition miR-145 regulates GSC migration and stemness by targeting CTGF which downregulates SPARC and SOX2 expression, respectively Therefore, miR-145 and CTGF are attractive therapeutic targets for anti-invasive treatment of astrocytic tumors and for the eradication of GSCs.

REGULATION OF CCN2 FIBROGENIC PATHWAYS BY EXOSOMAL MicroRNA

Li Chen[1]and David Brigstock[1,2]

[1]The Research Institute at Nationwide Children's Hospital, Columbus, OH, USA and [2]Department of Surgery, The Ohio State University, Columbus, OH, USA

Introduction: Hepatic stellate cells (HSC) are the principal fibrogenic cell type in the liver. After tissue injury, production of CCN2 increases in HSC and enhances the activation of these cells and their production of collagen. Here we show that CCN2 expression in activated HSC is regulated by intercellular delivery of miR-214 in nano-vesicular exosomes produced by other HSC. **Methods:** Exosomes were purified from HSC cultures by differential ultracentrifugation. The role of exosomes was determined by pre-treatment of donor HSC with GW4869 to inhibit neutral sphingomyelinase 2. Delivery of functional miR-214 to recipient HSC was established by luciferase activity after transfecting the cells with a luciferase reporter vector that was under the control of a wild type CCN2 3′-UTR or a mutant CCN2 3′-UTR lacking the miR-214 binding site. Co-culture experiments were established between donor HSC and either recipient HSC or hepatocytes to demonstrate exosomal transfer of miR-214. **Results:** MiR-214 was present in exosomes and its levels were increased by transfection of HSC with pre-mir-214. MiR-214 levels in exosomes but not in cell lysates were reduced by pre-treatment of the cells with GW4869. Co-culture of miR214-transfected donor HSC with CCN2 3′-UTR luciferase reporter-transfected recipient HSC resulted in miR-214-dependent and GW4869-dependent regulation of activity of the wild type, but not mutated, CCN2 3′-UTR. CCN2 3′-UTR activity in activated recipient HSC was more greatly inhibited in a GW4869-dependent manner by co-culture with freshly isolated donor HSC (high endogenous miR-214 levels) than with highly activated donor HSC (low endogenous miR-214 levels). Similar results were obtained for the human LX-2 HSC line. Mouse or human HSC delivered exosomal miR-214 to, respectively, primary mouse hepatocytes or human HepG2 cells resulting in reduced CCN2 3′-UTR activity in the target cells. **Conclusions:** MiR-214 is exported exosomally from HSC to other HSC or hepatocytes, suppressing CCN2 production in the recipient cells. These findings identify exosomal epigenetic regulation of CCN2 expression as a novel mechanism of regulating hepatic injury responses.

PATHOBIOLOGY OF CCN PROTEINS

PART II: METABOLISM AND DIFFERENTIATION

CELL TYPE-SPECIFIC REGULATION OF CCN2 EXPRESSION IN THE KIDNEY

Margarete Goppelt-Struebe

Department of Nephrology and Hypertension, Friedrich Alexander Universität Erlangen-Nürnberg, Erlangen, Germany

Increased expression of the profibrotic protein connective tissue growth factor (CCN2/CTGF) has been detected in injured kidneys, and elevated urinary levels of CCN2 are discussed as prognostic marker of chronic kidney disease. There is evidence

that epithelial cells lining the renal tubular system contribute to uptake and secretion of CCN2. However, there are functionally distinct types of tubular epithelial cells and their contribution to CCN2 secretion may vary. To address this question we analyzed primary cultures of human cells isolated from healthy parts of tumor nephrectomies. To approach the in vivo situation cells were cultured in insert wells to generate monolayers of polarized tubular cells.
The pro-fibrotic stimuli lysophosphatidic acid (LPA) and transforming growth factor β (TGF-β) were used to induce CCN2 secretion.
LPA activated CCN2 secretion in proximal tubular cells when applied from either the apical or the basolateral side as shown by immunocytochemistry. CCN2 was secreted exclusively to the apical side which would correspond to the urinary space in vivo. Signaling pathways activated by LPA included MAP kinase and Rho kinase signaling independent of the side of LPA application.
TGF-β applied from either side also stimulated CCN2 secretion primarily to the apical side with little basolateral release. Interestingly, TGF-β activation induced different signaling pathways depending on the side of TGF-β application. Smad signalling was exclusively activated from the basolateral side most prominently in cells of distal origin. Only part of these cells also synthesized CCN2 indicating that Smad activation per se was not sufficient for CCN2 induction. Further analysis in non-polarized cells revealed Smad-2 as regulator of CCN2 synthesis.
MAP kinases were involved in apical TGF-β-mediated activation of CCN2 synthesis in a subset of epithelial cells of distal origin. This subpopulation of distal tubular cells was also able to internalize recombinant apical CCN2, in addition to proximal cells which were the main cells to take up exogenous CCN2.
Analysis of polarized human primary epithelial cells thus showed that vectorial secretion of CCN2 depends on the cell type, the stimulus and the signaling pathway activated. In all conditions, CCN2 was secreted mainly to the apical side upon TGF-β and LPA treatment and therefore, likely contributes to increased urinary CCN2 levels observed in fibrotic kidneys in vivo.

POTENTIAL ROLE OF CCN3 IN TYPE 2 DIABETES

Jean Buteau[1] and Bernard Perbal [2]

[1] Alberta Diabetes Institute, University of Alberta, Edmonton, AB, Canada
[2] Laboratoire d'Oncologie Virale et Moléculaire, Université de Paris 7-D Diderot, Paris, France and International CCN Society

Type 2 diabetes is a metabolic disorder strongly associated with obesity. Both diabetes and obesity are increasing at an alarming rate and have reached epidemic proportions globally.
Pancreatic beta-cells secrete insulin to maintain blood glucose levels in a tight physiological range. Type 2 diabetes results from both insulin resistance and beta-cell dysfunction. The latter is characterized by a progressive deterioration of beta-cell mass and insulin secretion. However, the molecular mechanisms involved in beta-cell demise remain elusive.
We recently demonstrated that, in the pancreas, *Ccn3* expression is restricted to pancreatic islets. Moreover, our study characterized *Ccn3* as a novel transcriptional target of FoxO1, a transcription factor activated in insulin resistance. Consistently, CCN3 levels were elevated in animal models of diabetes/insulin resistance, as well as in obese humans. Functional studies revealed that CCN3 curtails both beta-cell proliferation and glucose stimulated insulin secretion.
We propose that CCN3 provides a molecular link between insulin resistance and beta-cell dysfunction. As such, CCN3 represents as a potential molecular target for diabetes treatment.

WISP2/CCN5 AS A REGULATOR OF PPARγ IN MESENCHYMAL CELLS

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WNT1-inducible-signaling pathway protein 2/Wisp2/CCN5 is highly expressed in mesenchymal cells. It is both a secreted and cytosolic protein. We have examined its role in adipogenesis and precursor cell commitment. BMP4-induced commitment of NIH3T3 fibroblasts to adipocytes with PPARγ activation is mediated through the dissociation of the Wisp2/Zfp423 cytosolic complex. This allows Zfp423 to enter the nucleus and activate PPARγ transcription. Wisp2/CCN5 is also a secreted protein and can inhibit PPARγ through unknown membrane receptor(s). To examine the effect of the secreted protein, we expressed a full-length and a truncated, non-secreted Wisp2 in NIH3T3 fibroblasts. Secreted, but not truncated Wisp2, activated the canonical Wnt pathway, increased phosphorylation of Lrp5/6, β-catenin and its nuclear targeting phosphorylation. It also inhibited Pparγ activation which was prevented by the Wnt antagonist Dickkopf-1. Thus, Wisp2/CCN5 exerts dual actions in adipogenesis, secreted Wisp2 activates canonical Wnt and maintains preadipocytes in an undifferentiated state while cytosolic Wisp2 regulates adipogenic commitment.

CCN5 IN UTERINE FIBROIDS AND OXYGEN INJURY IN LUNG: A PARADOX IN FUNCTION AND MECHANISM

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CCN5 is the oldest of the mammalian CCN proteins in evolutionary terms. It is comprised of only three of the four domains found on the other CCN family members—the carboxy-terminal domain is absent in CCN5. Considerable evidence demonstrates that CCN5 can inhibit a variety of cultured smooth muscle cell (SMC) types, including those from human fibroids, airways, and large arteries. CCN5 inhibits the proliferation of human leiomyoma cells implanted into immunocompromised mice. In the mouse carotid artery, CCN5 inhibits SMC proliferation following vascular injury. In contrast, convincing data from the Banerjee laboratory indicates that CCN5 plays an important role in promoting proliferation of estrogen-dependent breast cancer cells. These seemingly paradoxical results might be explained, at least in part, by the fact that SMC are mesenchymal in origin, whereas breast cancer cells are epithelial in origin. To examine this possibility, and to explore the role of CCN5 following oxygen-induced injury in the neonatal lung, we used neonatal wild-type (WT) C57/B6 mice. Five-day-old neonatal WT mice were exposed to room air (RA) or 90 % oxygen (hyperoxia) from days 5–13 of life, corresponding to the major period of murine alveolarization. On day 13, pups were sacrificed. The right lung was fixed, sectioned and used for immunofluoresence labeling. The left lung was isolated for protein analysis. Immunofluoresence co-staining showed that in WT

RA pups at day 13 of postnatal life, CCN5 was prominently expressed in type I alveolar cells (identified by labeling with T1 antigen). Co-labeling for Ki67 showed that CCN5-positive type I alveolar cells are actively proliferating. Co-labeling for surfactant protein C, a marker for type II alveolar cells, along with Ki67 and CCN5 showed that these cells are not proliferating and do not express CCN5. Western Blot analysis demonstrated that CCN5 expression is greatly reduced in hyperoxic lungs compared to normoxic lungs, and Ki67 analysis revealed greatly reduced proliferation of the type I alveolar cells. These *in vivo* data indicate CCN5 expression is high in proliferating alveolar type I cells during alveolarization and low or absent in non-proliferating alveolar epithelium. This is opposite from CCN5 expression in SMC and similar to the findings in breast cancer cells. Our data suggest that CCN5 may be a functional regulator of alveolar epithelial proliferation during normal lung maturation that is lost upon oxygen injury. These data support the hypothesis that CCN5 acts as an inhibitor of mesenchymal cell proliferation and as a stimulator of epithelial cell proliferation. However, we note that this is likely to an overly simplistic explanation, especially when considering that CCN5 is not associated with type II alveolar cell proliferation *in vivo*. Examination of many more mesenchymal and epithelial cell types is necessary to validate and refine this concept.

CCN4/WISP-1 ENHANCES CELL MIGRATION DURING SKIN WOUND HEALING

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Purpose: Previous studies have shown a strong involvement of the CCN family of matricellular proteins in skin wound healing process. Our preliminary study showed that CCN4/WISP-1 (CCN4) was strongly induced under the wound healing process compared to other CCN proteins. Therefore, this study aimed to investigate the role of CCN4 in wound healing.
Methods: CCN4 knock-out (KO) mice were generated and the wound healing process of the KO mice was compared to the wild-type (WT) mice in the dermal wound healing model. The gene expression levels of fibronectin (*Fbn)* and collagen type I (*Coll*) were analyzed by real time RT-PCR. Dermal fibroblasts were isolated from WT and KO mice for *in vitro* analysis of cell proliferation (MTS assay) and migration (Boyden Chamber assay). Moreover, cell proliferation and migratory capacity was also analyzed in human adult dermal fibroblasts (hADFs) following CCN4 knock-down by siRNA or over-expression by adenovirus vector.
Results: KO mice showed delayed wound healing, and the *Fbn* and *Coll* expression levels of KO mice were decreased compared to WT mice in wound healing process. Dermal fibroblasts from KO mice had significantly lower cell proliferation and migration capacity compared to those from WT mice. Moreover, knock-down of CCN4 using siRNA in hADFs significantly suppressed cell proliferation and migration. On the other hand, the results of overexpression of CCN4 gene using adenovirus vector revealed an enhanced cell migration, but no changes in cell proliferation of hADFs. Conclusion: CCN4, of which expression was highly enhanced during wound healing process, regulated the dermal wound healing by controlling the proliferation and migration of dermal fibroblasts.

ARE CCN FAMILY MOLECULES IMPLICATED IN THE REGULATION OF PIGMENTATION? THE EXAMPLE OF SYSTEMIC SCLEROSIS

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We have previously demonstrated that fibroblasts regulate pigmentation in vivo and in vitro. Indeed we were able to induce a caucasian-negroid switch in a mouse xenograft model. The major modification observed apart pigmentation was an increase in epidermal FGF-2. Among FGF2 targets we have hypothesized that proteins of the CCN family which are implicated in fibrosis such as CCN2 (CTGF) may be also implicated in the regulation of pigmentation. Indeed CCN3 is decreased in melanocytes a finding recently described by our group in vitiligo.
In systemic sclerosis (SSc), a disease characterized by an excessive production of collagen by fibroblasts, 43 % of patients present hypo or hyperpigmentary changes. CCN2 (CTGF) has been investigated because of its pro-fibrotic properties. We looked at pigmentary changes in correlation with CCNs and FGF2 in SSc patients and normal controls. Our data indicate that inter-individual variations in CCN expression are not correlated with phototype or age in the control group. On the contrary, our preliminary data on SSc patients point out a link between expression of cutaneous CCNs, FGF-2 and epidermal pigmentation. Those findings reinforce the concept of a dermal regulation of epidermal pigmentation in a subset of cutaneous dermal disorders and of a major role of FGF-2.

ROLE OF CCN2 IN DEVELOPMENTAL AND PATHOLOGICAL ANGIOGENESIS IN THE RETINA

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Angiogenesis and fibrosis play an important role in tissue development and regeneration and wound healing. Both processes occur either concurrently or sequentially in many disease states where pathological angiogenesis initiates extracellular matrix (ECM) remodeling, abnormal vessel formation and scarring. Connective tissue growth factor, CCN2/CTGF, is best known for its pro-fibrotic and profibrogenic activities. Like other matricellular proteins, CCN2 bridges the functional divide between structural macromolecules and growth factors, cytokines, proteases, and other related proteins, by virtue of its ECM-like structural features and its multifaceted activities including modulation of cell motility, adhesion, proliferation, epithelial-mesenchymal cell transition and production of endothelial basement membrane components. As such, this molecule is a prime candidate for the modulation of blood vessel formation during development and diseases.
Here we focused on the role of CCN2 in retinal vessel development and repair of damaged retinal vessels following hyperoxic injury using loss- and gain-of-function approaches. We examined the expression and function of CCN2 in the retinal vasculature which develops postnatally in mice and in the model of oxygen-induced retinopathy (OIR) which recapitulates the formation of abnormal blood vessels in the eye as a result of ischemia.
CCN1 promoter-GFP reporter mice were used to determine the tissue distribution of CCN2. CCN2 promoter-driven GFP, which recapitulates

endogenous CCN1 expression in mice, showed a dynamic but transient expression of the transgene with maximum expression during the formation of the primary and secondary capillary plexuses. Endothelial cells, pericytes, Muller cells and astrocytes were major sources of CCN2. CCN2 was abnormally increased and localized within neovascular tufts in the mouse eye with OIR. Ectopic expression of the CCN2 gene through lentivirus -mediated gene transfer further exacerbated neovascularization in the retina while lentivirus-mediated loss-of-function or -suppression of CCN2 significantly reduced ischemia-induced neovescular growth in mice. The neovascular effects of CCN2 were mediated, at least in part, through increased expression and activity of matrix metalloproteinase (MMP)-2. In cultured cells, CCN2 activated MMP-2 promoter through increased expression and tethering of the p53 transcription factor to a highly conserved p53 binding sequence within the MMP-2 promoter. Concordantly, the neovascular effects of CCN2 were suppressed by p53 inhibition which culminated into reduced enrichment of the MMP-2 promoter with p53 and decreased MMP-2 gene expression. These data identified new gene targets and downstream effectors of CCN2 and provided the rationale basis for targeting the p53 pathway to curtail the effects of CCN2 on neovessel formation associated with ischemic retinopathy.

PATHOBIOLOGY OF CCN PROTEINS

PART III: FIBROSIS

CCN1 FUNCTIONS IN LIVER FIBROSIS AND CANCER

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Liver fibrosis occurs as a wound-healing response to chronic hepatic injuries irrespective of the underlying etiology and may progress to life-threatening cirrhosis. CCN1 is accumulated in hepatocytes of human cirrhotic livers, and Ccn1 expression is upregulated in mouse models upon liver injuries. Ccn1 functions to inhibit liver fibrogenesis induced by either carbon tetrachloride intoxication or bile duct ligation and promote fibrosis regression. CCN1 acts by triggering cellular senescence in activated hepatic stellate cells and portal fibroblasts by engaging integrin alpha6beta1 to induce reactive oxygen species accumulation through the RAC1-NADPH oxidase 1 enzyme complex, whereupon the senescent cells express an antifibrosis genetic program. Mice with hepatocyte-specific Ccn1 deletion suffer exacerbated fibrosis with a concomitant deficit in cellular senescence, whereas overexpression of hepatic Ccn1 reduces liver fibrosis with enhanced senescence. Furthermore, tail vein delivery of purified CCN1 protein accelerates fibrosis regression in mice with established fibrosis. While CCN1 is accumulated upon liver injury to dampen fibrosis, it also inhibits incipient tumorigenesis.
These findings reveal a novel integrin-dependent mechanism of fibrosis resolution in chronic liver injury and identify the CCN1 signaling pathway as a potential target for therapeutic intervention.

SKIN PROGENITOR CELLS CONTRIBUTE TO BLEOMYCIN-INDUCED SKIN FIBROSIS

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Background
The origin of the cells that contribute to skin fibrosis is unclear. Herein, we assess the contribution of sox2-expressing skin progenitor cells to bleomycin-induced skin scleroderma.
Methods
We subject wild type mice and mice in which CCN2 is deleted in sox2-expressing cells to bleomycin-induced skin scleroderma. We also conduct lineage tracing analysis to assess whether cells expressing sox2 are recruited to fibrotic lesions in response to scleroderma
Results
In response to bleomycin, sox2-positive/α smooth muscle actin-positive cells are recruited to fibrotic tissue. Conditional CCN2 knockout mice in which CCN2 is deleted in sox2-expressing cells exhibit resistance to bleomycin-induced skin fibrosis. Collectively, these results indicate that CCN2 is required for the recruitment of progenitor cells and that CCN2-expressing progenitor cells are essential for bleomycin-induced skin fibrosis. Lineage tracing using mice in which a tamoxifen-dependent cre recombinase is expressed under the control of the sox2 promoter confirm that progenitor cells are recruited to the fibrotic lesion in response to bleomycin, but not in CCN2-knockout mice. CCN2 was required for the ability of serum to induce α smooth muscle actin mRNA expression in skin progenitor cells.
Conclusion
These data indicate that sox2-positive skin progenitor cells are required for bleomycin-induced skin fibrosis and that CCN2 is required for their recruitment to the fibrotic lesion. Targeting stem cell recruitment or CCN2 may therefore represent useful targets in combating fibrotic skin disease.

CCN2 AS A TARGET FOR ANTIFIBROTIC THERAPY IN KIDNEY DISEASE

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Kidney fibrosis is the common endpoint of chronic kidney disease, irrespective of its aetiology. Currently no effective therapy exists to reduce kidney fibrosis. CCN2 , also known as “Connective Tissue Growth Factor” or “CTGF”, appears to be an interesting candidate for anti-fibrotic drug targeting, because it holds a central position in the development of kidney fibrosis. Although a specific CCN2 receptor does not appear to exist, CCN2 can bind to and signal through a number of known transmembrane receptors including integrins, LRP1 and −6, EGFR, and TrkA. In addition, CCN2 might modify signalling activity and cross-talk of multiple pathways through interaction with extracellular matrix and a variety of factors that are critically involved in pro- and anti-fibrotic responses, including TGFβ and BMPs, IGF, and VEGF. On the other hand, CCN2 is important in development and regeneration of skeletal structures, its expression is associated with a stable plaque phenotype in atherosclerosis, and over-expression of CCN2 as found to protect the heart in overload hypertrophy and ischemia reperfusion models. Still, numerous experimental and clinical studies lowering CCN2 bioavailability have shown promising results with minimal if any adverse side effects.
It remains to be established whether specific targeting of critical cell types and pathways might provide additional benefits in designing anti-CCN2 therapies.
While numerous experimental and clinical studies lowering CCN2 bioavailability have shown promising results with minimal adverse side effects, overexpression of CCN2 protected the heart in overload hypertrophy and ischemia reperfusion models.

NOVEL MECHANISMS INVOLVED IN THE DEVELOPMENT OF FIBROSIS ASSOCIATED TO DUCHENNE MUSCULAR DYSTROPHY

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Muscular dystrophies are characterized by a decrease of skeletal muscle mass and force and an increase in fibrosis. Connective tissue growth factor (CTGF/CCN2), transforming growth factor type-β (TGF-β and angiotensin–II (Ang-II) are overexpressed in muscular dystrophies and correlates with the severity of fibrosis in many diseases. However, the role of these factors in Duchenne Muscular Dystrophy (DMD) and associated fibrosis remains unknown.

Using two independent approaches we found that mdx mice with reduced CTGF availability do indeed have less severe muscular dystrophy. Mdx mice with hemizygous CTGF-deletion (mdx-Ctgf +/−), and mdx mice treated with a neutralizing anti-CTGF monoclonal antibody (FG-3019), performed better in an exercise endurance test, had better muscle strength in isolated muscles, and reduced skeletal muscle impairment, apoptotic damage and fibrosis. Transforming growth factor type-β (TGF-β signaling remained unaffected during CTGF suppression. Moreover, both mdx-Ctgf+/−, and FG-3019 treated mdx mice had improved grafting upon intramuscular injection of dystrophin positive satellite cells.

Acting via the Mas receptor, Angiotensin-1-7 (Ang-(1-7)) is part of RAS, with the opposite effect to that of (Ang-II). We hypothesized that the Ang-(1-7)/Mas receptor axis might protects chronically damaged tissues as the present in skeletal muscle of the DMD mouse model mdx. TGF-b injection in wl skeletal muscle induce expression of fibrosis and CTGF/CCN2, but this is abolished by co-injection with Ang-1-7. Infusion or oral administration of Ang-(1-7) in the mdx normalized skeletal muscle architecture, decreased local fibrosis and improved muscle function in vitro and in vivo. These positive effects were mediated by the inhibition of TGF-β Smad signaling. Mdx mice infused with Mas antagonist (A-779) and mdx deficient for the Mas receptor showed highly deteriorated muscular architecture, increased fibrosis and TGF-β signaling with diminished muscle strength.

These results will be discussed in the context of DMD and possible therapeutics approaches to improve quality of life and delay death in individuals with DMD.

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ABNORMALLY DIFFERENTIATING KERATINOCYTES IN SYSTEMIC SCLEROSIS EPIDERMIS SHOW ENHANCED SECRETION OF CCN2

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Systemic sclerosis (SSc) is a severe disease of unknown aetiology characterised by cellular injury and activation in early stage, followed by autoimmunity and fibrosis. Much of the work is focused on the fibroblasts however, keratinocytes are known to be able to secrete chemo-attracting agents as well as growth factors influencing phenotype and proliferation rate of fibroblasts. Moreover, appropriate epithelial-mesenchymal interactions are essential for homeostasis and tissue repair. We have recently shown that SSc epithelial cells exhibit an activated phenotype similar to wound healing. Therefore, we decided to further characterise SSc epidermis and look for evidence that in SSc injured epidermal cells are releasing factors capable of promoting fibrosis. Here we demonstrate that SSc epidermis is significantly thickened than control epidermis ($p<0.05$) and contain hypertrophic cells in basal ($p<0.05$) and spinous layers ($p<0.05$). In addition, we observe higher number of keratinocytes present in SSc epidermis ($p<0.005$) however, expression of the proliferation marker ki-67 shows only trend towards increase. We also show that SSc epidermis has altered expression of differentiation markers involucrin, loricrin and filaggrin. Furthermore, immunohistochemistry performed on SSc sections demonstrate nuclear translocation of phosphorylated Smad2/3 in SSc epidermis, further strengthening evidence from our previous report showing wound healing-like phenotype of SSc keratinocytes. We also reveal that media conditioned with epidermal explants of SSc patients ($n=8$) release significantly increased levels of CCN2 when compared to healthy controls ($n=8$) ($p<0.05$). Moreover, staining of skin sections results have confirmed higher levels of CCN2 in SSc the epidermis, dermis and around blood vessels, when compared to healthy skin. Interestingly early SSc skin dermal staining was limited to the papillary dermis at the epidermal-dermal junction, while in established disease positive staining was observed also in the lower dermis. Additionally, relative levels of CCN2 mRNA in the epithelial blister sheets was three fold increased ($p<0.05$). Our results demonstrate that SSc epidermis has altered expression of differentiation markers suggesting changes in terminal differentiation and signalling. Moreover, SSc epidermis provides a potential source of CTGF in SSc skin and therefore can contribute to SSc skin fibrosis.

INVESTIGATING THE ROLE OF MRTF-A IN SYSTEMIC SCLEROSIS

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Background

MRTF-A is a 120 kDa transcription factor widely expressed and normally sequestered in the cytosol by binding to G actin. Following actin polymerisation downstream of Rho signalling, or mechanosensing, MRTF-A is released and functions as a signalling molecule partnering serum response factor (SRF), influencing gene expression via CARG elements. Genes expressing CARG like elements induced by MRTF-A/SRF include CTGF and type I collagen. MRTF-A is also implicated in vascular remodelling and epithelial to mesenchyme transition (EMT). The MRTF-A/SRF axis is highly relevant to fibrosis in systemic sclerosis (SSc).

Methods

MRTF-A signal transduction was studied in healthy control and SSc fibroblasts. The MRTF-A/SRF small molecule inhibitor CCG1423 was used to block MRTF-A in vitro. SSc fibrosis responses were modelled by collagen gel contraction, CTGF, and type I collagen expression. MRTF-A signalling was assayed by Western blotting of nuclear and cytoplasmic extracts. Wound healing and fibrosis was studied in an MRTF-A knockout mouse (Ollsen lab) and wild type controls. Immunochemistry looking for nuclear localisation of MRTF-A was used to determine presence of active signalling in SSc involved skin biopsy material and healthy control tissue.

Results

SSc fibroblasts showed enhanced nuclear localisation of MRTF-A at 8 h following exposure to TGFβ (4 ng/ml) not seen in healthy control fibroblasts. Immunochemistry of SSc skin biopsy material revealed

enhanced nuclear localisation in dermal fibroblast like cells, keratinocytes within the epidermis, as well as in perivascular cells (pericytes).
Following excisional wounding (4 mm punch biopsy, basal wound area 12.6 mm^2) MRTF-A mice wounds failed to close normally and increased in size during days 1-7, wound area decreasing by day 11. When compared to wild type controls MRTF-A knockout wounds were enlarged at day 7 (wild type area 6 mm^2, knockout area 12.4 mm^2, $p<0.03$), and at day 11 (wild type area 0.42 mm^2, knockout area 3.4 mm^2, $p<0.01$). Day 11 wounds were extracted and found to abnormal showing reduced scar formation, and abnormal vasculogenesis. Small blood vessels within the granulation tissue were dilated, and exhibited extravasation of red blood cells. Gel contraction by wild type fibroblasts was enhanced by TGFβ and blocked by CCG1423 1 μM (basal conditions mean gel mass 0.176 g, TGFβ treated 0.118 g, TGFβ+CCG1423 0.238 g, $p<0.002$). Dermal fibroblasts from MRTF-A knockout mice showed reduced basal gel contraction, and impaired response to TGFβ,(basal conditions mean gel mass 0.349 g, TGFβ treated 0.259 g, TGFβ+CCG1423 0.313 g ($p<0.05$ basal vs wild type). Studies of belomycin induced skin fibrosis in MRTF-A −/− mice are ongoing.

Conclusions

MRTF-A signalling is abnormal in SSc involved skin, enhanced in multiple cell types, as well as in SSc fibroblasts cultured in vitro. MRTF-A knockout mice fail to contract wounds adequately and show reduced scar formation, as well as abnormal vasculogenesis. Multiple pro-fibrosis pathways converge on MRTF-A including response to stiffness of the extracellular matrix, profibrosis growth factor stimulation, as well as transition of epithelial cells and perivascular cells to mesenchymal cells. CCG1423 and its derivatives may be potential anti-fibrotics to benefit SSc fibrosis.

LONG-TERM PERFORMANCE OF A HIGH DEMAND REPETITIVE TASKS INDUCES BOTH INCREASED SERUM AND MUSCLE CTGF/CCN2 AND MOTOR DECLINES

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Several serological compounds, including Connective Tissue Growth Factor (CTGF) has been identified as a noninvasive marker of fibrosis and fibrosis progression. A key feature of long-term performance of repetitive tasks at high strain rates is muscle fibrosis and reduced grip strength, although the relationship between these two features is still under investigation. Here, we examined serum and muscle CTGF and its relationship to grip strength, in a unique operant rat model of work-related musculoskeletal disorders. Young adult, Sprague-Dawley, female rats learned (trained) and then performed either a high repetition low force task (HRLF) or a high repetition high force task (HRHF), each with a reach rate of 4 reaches/min, and pulling forces of 0.23 and 1.07 N, respectively (15 % and 60 % of the rats' maximum pulling force). They performed these tasks for 2 h/day, in four 30 min sessions/day, 3 days/week, for 18 weeks. Results were compared to control rats. ELISA analysis of serum showed that CTGF was increased in serum of 18-week HRLF and 18-week HRHF rats, compared to controls ($p<0.05$ and $p=0.01$, respectively), indicative of an exposure-dependent increase in serum CTGF. Both ELISA and western blot analysis of flexor digitorum muscle lysates showed elevated CTGF in 18-week HRLF and 18-week HRHF muscles, compared to controls ($p=0.03$ and $p<0.01$, respectively), with greater increases in HRHF rats. Western blot analysis also showed increased levels of mature collagen type I in the 18-week HRHF muscles (but not in 18-week HRLF muscles), a clear indication of exposure-dependent increases in muscle fibrosis. Immunohistochemistry revealed epimyseum thickening and increased of CTGF-positive fibroblasts in and around muscle fibers of each task group, compared to control rats, and that that HRHF task induced greater changes than the HRLF task. Grip strength declined in each task group, although the decrease was greater in the HRHF rats. The increased CTGF in muscle and serum correlated with a persistent decrease in grip strength in HRHF rats ($p<0.001$), but only serum CTGF correlated with decreased grip strength in HRLF rats ($p=0.001$). We suggest that fibrosis may be a critical component in repetitive motion injuries that deserves further study as a potential target for therapeutic intervention, and that serum CTGF may serve as a serum biomarker of fibrosis progression in work-related musculoskeletal disorders.

CCN BIOLOGY:

FROM GENES TO PROTEINS

MECHANISMS OF INCREASED GINGIVAL COLLAGEN ACCUMULATION BY CCN2

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Elevated levels of CCN2 have long been associated with fibrotic forms of drug-induced, hereditary, and idiopathic human gingival overgrowth. Although much is known regarding tissue-specific regulation of CCN2 in human gingival fibroblasts, the mechanisms by which CCN2 promotes gingival overgrowth and fibrosis are unknown. Here we investigate the possible role of CCN2 regulation of lysyl oxidases in promoting collagen accumulation. Lysyl oxidases are critically required extracellular enzymes which participate in collagen maturation and deposition.
Primary human gingival fibroblasts from at least three different donors were cultured and treated with recombinant human CCN2 in the presence or absence of a peptide previously shown to block CCN2-stimulated collagen accumulation, and expressions of lysyl oxidase isoforms were analyzed by Western blotting, normalized to β-actin. Only lysyl oxidase-like-2 (LOXL2) was up-regulated by CCN2, and blocked by the peptide. To determine whether LOXL2 mediates CCN2-stimulated collagen accumulation, CCN2 was knocked-down by shRNA lentivirus technology and cell layer collagen accumulation assessed by a Sirius Red-based assay. Data indicate that collagen accumulation was inhibited below levels of non-treated cells by LOXL2 shRNA. Moreover, crystal violet staining of cell layers suggested that cell numbers were much lower than controls in LOXL2 shRNA cells. Addition of rLOXL2 protein to human gingival fibroblast cultures restored collagen accumulation and crystal violet staining to normal. This led to the hypothesis that LOXL2 could have a role in human gingival fibroblast proliferation, and not only in collagen maturation. LOXL2 shRNA knockdown cells and empty virus controls were therefore subjected to short-term (CyQuant) and long-term (growth curve) proliferation assays in the presence and absence of recombinant CCN2. Data indicate that LOXL2 knockdown cells in the presence or absence of CCN2 proliferated slowly compared to corresponding controls, with no signs of obvious

toxicity measured by lactate dehydrogenase (LDH) assays of media samples, and visual inspection. Finally, pharmacologic inhibition of LOXL2 enzyme activity similarly inhibited primary human gingival fibroblast proliferation.
These findings identify a novel mechanism by which CCN2 promotes gingival fibrosis that involves both proliferative and collagen maturation activities of LOXL2. A comprehensive model for TGF-β stimulated CCN2 and LOXL2 dependent collagen accumulation in human gingival fibroblasts will be presented. This model has undergone initial testing for therapeutic approaches to address gingival overgrowth, and preliminary studies from mouse in vivo work will be presented. This research was supported by NIH NIDCR R01 DE011004.

CCN EXPRESSION AND FRAGMENTATION IN THE LUNGS

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CCN proteins are matricellular proteins that play an important role in tissue development and remodeling. In the lungs, CCNs have been implicated in several physiological alterations and diseases including ventilator- and hyperoxia-induced injuries, pulmonary fibrosis and cancer. Most available data result from cellular and animal models, but observations in humans are still scarce.
We combined *in vivo* and *in vitro* approaches in order to better delineate how CCN expression is altered in diseases including non-small cell lung cancer (NSCLC) and chronic obstructive pulmonary disease (COPD). CCN expression was examined by RT-qPCR in frozen tissue samples from about 130 patients suffering NSCLC associated or not with COPD. CCN1, 2 and 3 were found down-regulated in cancerous tissues compared to non-cancerous tissues . Among the five CCN mRNAs (CCN1-5) detected in non-cancerous tissues, only CCN1 mRNA displayed a significant upregulation in samples from COPD patients compared to control smokers. Cigarette smoke (CS) is accepted as the most important risk factor for the development of NSCLC and COPD. Thus, we examined the impact of CS exposure on CCN expression in mice models and also *in vitro*, using human cell lines. Balb/C mice were exposed to cigarette smoke during 4 days (acute exposure) or 8 weeks (chronic exposure) and RNA expression of the CCN1-5 was measured in whole lung extract. Acute CS exposure triggered a significant decrease in the RNA levels of CCN3 but was without effect on expression of the other CCNs. By contrast, three CCNs (CCN1, 3, 4) were found down-regulated in the lungs of mice submitted to chronic smoke exposure. Human bronchial cells (BEAS-2B & NCI-H292) were also exposed to CS extract for 6 h. However, this treatment did not alter CCN expression. Taken together, our results suggest that CS has little effects *per se* on whole lung expression of the genes encoding CCNs. These proteins are sensitive to proteolysis *in vitro* and, in a first attempt to determine their status *in vivo,* we examined their fragmentation pattern in aspirates from patients in intensive care. All CCNs were found fragmented in the bronchial aspirates. Fragmentation may be due to action of bronchial and/or inflammatory proteases as evidenced by *in vitro* studies.
In conclusion, our observations indicate that besides gene regulation, CCN proteolysis could be an important mechanism controlling expression and function of these matricellular proteins in the lungs.

NEW FUNCTIONAL ASPECTS OF KNOWN MOLECULES AS CCN2 PARTNERS

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As generally recognized, CCN2 interacts with a vast number of biomolecules and modulates their functionalities. This also suggests that, by analyzing the behavior of these molecules from the viewpoint of CCN2 cofactors, their novel functional aspects can be occasionally uncovered.
Low-density lipoprotein receptor-related protein 1 (LRP-1) is a huge cell surface molecule with multiple functions under the interaction with more than 40 ligands. This 600-kDa molecule plays critical roles in a variety of tissues and organs including growth plate cartilage. CCN2 is also known to be indispensable for the integrated development of the growth plate, and direct interaction between LRP-1 and CCN2 was already reported in 2001. Nevertheless, the molecular function of LRP-1 in the growth plate in relation to CCN2 remained to be clarified.
CCN2 is produced predominantly in a restricted population of growth plate chondrocytes in the pre-hypertrophic layer. However, CCN2 protein is distributed broadly from proliferating to late hypertrophic layers around the producers; whereas it is absent in the resting layer. Our investigation on the interaction between CCN2 and LRP-1 during endocytotic trafficking in human chondrocytic HCS-2/8 cells revealed a novel property of LRP-1 as a CCN2 transporter across the layer of chondrocytes. This transcytosis is dependent on clathrin, and colocalization of CCN2 with early and recycling endosomal markers is observed during the intracellular transport. Knocking down of LRP-1 or addition of an LRP-1 antagonist diminished the intracellular uptake of CCN2, indicating that this event is dependent on LRP-1. Of note, the direction of CCN2 transport in the growth plate cartilage appears to be regulated by the endogenous LRP-1 antagonist. These findings are also useful in investigating the molecular behavior of CCN2 in any microenvironment where LRP-1 is present.
Most recently, we have found that platelet growth factor receptor-like (PDGFRL) protein as another CCN2 partner. Although a few reports suggest this protein as a tumor suppressor, our investigation is revealing a role of PDGFRL as a molecular decoy for CCN2. Current findings on this PDGFRL-CCN2 connection are going to be introduced as well.

MATRICELLULAR PROTEIN CCN1 REGULATES CARDIOMYOCYTE APOPTOSIS IN MICE WITH STRESS-INDUCED CARDIAC INJURY

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Expression of extracellular matrix protein CCN1 is induced in end-stage ischemic cardiomyopathy in humans, and after cardiac ischemia and reperfusion in experimental animal models. Despite its well-documented angiogenic activities, CCN1 increases the cytotoxicities of the tumor necrosis factor family cytokines, which promotes apoptosis in

fibroblasts. We aimed to determine the physiological function of CCN1 in an injured heart. To assess the function of CCN1 *in vivo*, knock-in mice carrying the apoptosis-defective mutant allele *Ccn1-dm* were tested in an isoproterenol (ISO)-induced myocardial injury model (100 mg/kg/day of subcutaneously injected ISO for 5 days). Compared with wild-type mice, $Ccn1^{dm/dm}$ mice were remarkably resistant to ISO-induced cardiac injury: They showed no post-treatment cardiomyocyte apoptosis or myocardial tissue damage. ISO cardiotoxicity is dependent on Fas ligand (FasL) and its downstream signaling. Using primary cultures of cardiomyocytes isolated from rats, we demonstrated that CCN1 sensitized FasL-mediated apoptosis by engaging its cell surface receptor integrin $\alpha_6\beta_1$ and upregulating intracellular reactive oxygen species (ROS), which activated mitogen-activated protein kinase p38, and increased cell-surface Fas expression. In conclusion, CCN1 is a critical pathophysiological regulator that mediates cardiomyocyte apoptosis during work-overload-induced cardiac injury. CCN1 increases cellular susceptibility to Fas-induced apoptosis by increasing ROS and cell-surface Fas expression.

IMPLICATIONS OF CCN2 IN INFARCT HEALING AND MYOCARDIAL REMODELING AFTER MYOCARDIAL INFARCTION

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Adequate scar healing is critical after myocardial infarction (MI). However, prolonged activity of myofibroblasts may cause excessive myocardial fibrosis leading to impaired cardiac function. Myocardial expression of CCN2 is substantially increased in ischemic heart failure. Yet, the role of CCN2 in healing of MI and in regulation of myocardial fibrosis is still poorly understood. Thus, the objective of this study was to investigate the role of CCN2 in infarct healing and myocardial fibrosis following MI.
MI was induced by ligation of the left coronary artery in transgenic mice with cardiac-restricted overexpression of CCN2 (Tg-CCN2) and in non-transgenic control (NTC) mice. Primary cardiac fibroblasts were isolated from NTC mice and stimulated with or without recombinant human CCN2 (rhCCN2) (250 nmol/L) and/or TGFβ-1 (2.5 μg/ml) after first passage.
Area of necrosis 24 h after induction of MI was similar in Tg-CCN2 and NTC mice. The collagen contents of the infarct region was higher and increased more rapidly in Tg-CCN2 mice than in NTC mice (day 5 post-MI collagen contents in Tg-CCN2 vs. NTC mice: 21.3±0.4 vs. 16.6± 0.8 μg/mg dry weight, $P<0.01$). Interestingly, this difference was reversed at 42 days post-MI, a time point at which collagen contents of the infarct region were lower in Tg-CCN2 mice versus NTC mice (88.9±8.6 vs. 114.3±6.1 μg/mg dry weight, $P<0.05$). The enhanced deposition of collagen in the differentiating scar tissue of Tg-CCN2 mice reflected in a lower incidence of myocardial rupture compared with that in NTC mice (2/41 vs 10/39, $P=0.01$). Interestingly, rhCCN2 inhibited TGFβ-induced differentiation of primary cardiac fibroblasts towards the myofibroblast phenotype as reflected by reduced upregulation of α-smooth muscle actin. Impaired myofibroblast differentiation following exposure to rhCCN2 also manifested in both reduced myofibroblast migration and proliferation. The mechanism of the reduced sensitivity of cardiac fibroblasts to TGFβ-induced myofibroblast transformation after prolonged exposure to rhCCN2 was reduced TGFβ-stimulated phosphorylation of Smad2[Ser465/467] due in part to downregulation of total SMAD2 levels.
In a cohort of patients with acute ST-elevation MI ($n=42$) admitted to hospital for percutaneous coronary intervention serum-CCN2 levels (s-CCN2) were monitored and related to scar tissue and left ventricular function assessed by cardiac MRI after 12 months. Increase of s-CCN2 levels after MI was associated with smaller scar and improved left ventricular ejection fraction 1 year after MI.
In conclusion, this study shows that infarct healing is enhanced in Tg-CCN2 mice and that increased serum CCN2 levels in patients after MI is associated with attenuated left ventricular remodeling and improved function 1 year after the insult. CCN2 appears to limit collagen deposition and myocardial fibrosis in ischemic heart failure by reducing the sensitivity of cardiac fibroblasts to TGFβ.

BIOLOGICAL FUNCTIONS OF CCN PROTEINS IN DEVELOPMENT

ROLES OF CCN2 AND CCN3 IN SKELETOGENESIS

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CCN family member 2/connectivie tissue growth factor (CCN2/CTGF) has been suggested to be an endochondral ossification genetic factor that has been termed "ecogenin", because *in vitro* studies revealed that CCN2 promotes the proliferation and differentiation of growth-plate chondrocytes, osteoblasts, and vascular endothelial cells and the formation of osteoclasts, all of which cells play important roles in endochondral ossification. For confirmation of this hypothesis, transgenic mice over-expressing CCN2 in cartilage were generated. The results proved the hypothesis; i.e., the over-expression of CCN2 in cartilage stimulated the proliferation and differentiation of growth-plate chondrocytes, resulting in the promotion of endochondral ossification. In addition to its "ecogenin" action, cartilage-specific overexpression of CCN2 in the transgenic mice was shown to protect against the development of osteoarthritic changes in aging articular cartilage, suggesting its anti-aging (chondroprotective) role in articular cartilage. CCN2 also had been shown to promote intramembranous ossification, regenerate cartilage and bone, and induce angiogenesis *in vivo*. For understanding of the molecular mechanism underlying such multifunctional actions, we have searched for binding partners of CCN2. ECMs such as fibronectin and aggrecan, growth factors including BMPs and FGF2 and their receptors such as FGFR1 and 2, as well as CCN family members, CCN2 and CCN3, were shown to bind to CCN2. Regarding the interaction of CCN2 with some of them, various binding modules in the CCN2 molecule have been identified. Therefore, the numerous biological actions of CCN2 would depend on what kinds of binding partners and what levels of them are present in the microenvironment of different types of cells, as well as on the state of differentiation of these cells. Through this mechanism, CCN2 would orchestrate various signaling pathways, acting as a signal conductor to promote harmonized skeletal growth and regeneration.
In general, CCN3 has been believed to have opposite actions to those of CCN2. Actually, we previously found that CCN3 inhibited proteoglycan synthesis in growth plate chondrocytes. However, CCN3 stimulated aggrecan gene expression and proteoglycan synthesis in epiphyseal chondrocytes and expression of articular cartilage phenotype such as tenascin-C expression by the cells, suggesting that CCN3 direct the

differentiation of chondroblasts toward articular chondrocytes. Interestingly, CCN2-CCN3 heterodimer strongly promoted aggrecan gene expression in chondrocytic HCS-2/8 cells. To investigate the role of CCN3 in cartilage, we also generated transgenic mice over-expressing CCN3 in cartilage. The mice showed dwarfism. Analysis of the skeletal phenotype suggests that CCN3 overexpression in cartilage delayed eondochondral ossification.

In conclusion, CCN2 promotes skeletogenesis by binding various growth factors and their receptors including CCN3, which also play positive/negative roles in skeletogenesis.

CARTILAGE-SPECIFIC OVEREXPRESSION OF CCN3 MODULATES ENDOCHONDRAL BONE FORMATION

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CCN family protein 3/ Nephroblastoma OVerexpressed (CCN3/NOV) deletion mice (*Ccn3*^{del3}) in which the 3rd domain is deleted showed abnormal endochondral ossification, indicating the important role of CCN3 in skeletal development. In our previous reports we showed that CCN2 enhances endochondral ossification, and CCN3 modifies production of cartilaginous extracellular matrices by binding to CCN2. Here we generated transgenic mice in which the *Ccn3* gene is expressed specifically under the type II collagen (*Col2a1*) promoter and analyzed their long bones. Embryonic long bone from *CCN3*^{Col2a1tg} mice showed thick and short bony part in skeletal preparation, and analysis of the skeletal phenotype by micro CT of femora from adults *CCN3*^{Col2a1tg} mice showed decreased bone volume, bone surface density, trabecular thickness, and trabecular number. *In situ* hybridization of E15.5 tibiae from *CCN3*^{Col2a1tg} mice showed delayed cartilage development and less osteoblastic markers. Furthermore, histological analysis of embryonic tibia from *CCN3*^{Col2a1tg} showed a reduced number of TRAP-positive osteoclasts and CD31-positive vascular endothelial cells in the spongiosa. Our data indicates that CCN3 overexpression in cartilage delayed endochondral ossification, possibly by inhibited vascular invasion and impaired osteogenesis by reducing osteoblastogenesis. CCN3 overexpression in cartilage may impair osteogenesis by modifying replacement of cartilage to bone during endochondral ossification.

EVALUATING THE REGENERATIVE EFFECT OF CCN2 INDEPENDENT MODULES ON CHONDROCYTES IN VITRO AND OSTEOARTHRITIS MODELS IN VIVO

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CCN2 (CCN family member 2/connective tissue growth factor) is known to promote the regeneration of articular cartilage. It is comprised of 4 highly interactive modules; IGFBP, VWC, TSP1 and CT. This study aims to assess the effects of these modules independently and their combinations on chondrocytic cells *in vitro* and damaged cartilage *in vivo*, especially in relation to full length CCN2. In this context, direct molecular interaction between these modules was also evaluated.

Methods

Effect of a single module, or their different combination, was evaluated with human chondrocytic HCS-2/8 cells. The cellular phenotype was estimated by the gene expression of chondrocytic markers. Proteoglycan synthesis and proliferation were evaluated by [^{35}S]-sulfate and [^{3}H]-thymidine incorporation assays, respectively. Cartilage regeneration *in vivo* was evaluated by using 2 rat models simulating osteoarthritis via surgical or chemical intervention. Physical interaction of 2 modules was kinetically examined by a surface plasmon resonance (SPR) methodology.

Results

Functional analysis *in vitro* revealed a biological activity even stronger than the full length CCN2 in a particular module. Interestingly, mixed application of all 4 modules almost reconstructed the bioactivity to the level of the full length. The result of cartilage regeneration experiments *in vivo* was also consistent with the findings obtained *in vitro*. SPR analysis uncovered significant interaction of 2 modules and full-length CCN2.

Conclusion

These results indicate independent bioactivity of each module for cartilage regeneration and suggest unknown means of binding to construct a possible tetramodular complex that is functionally comparable to the full length CCN2.

ROLE OF CCN3 IN OSTEOBLAST DIFFERENTIATION AND BONE REGENERATION

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We reported that CCN3 inhibits osteoblast differentiation by interacting with BMP and Notch signaling using osteoblastic cells lines (BBRC 345:567–573,2007, BBRC 368: 808–814,2008), but the role of CCN3 in bone regeneration has not been well elucidated. We therefore investigated the role of CCN3 in bone regeneration. *Ccn3* gene was identified as a highly expressed gene at the early phase of bone regeneration in a mouse bone regeneration model by microarray analysis. We confirmed the upregulation of *Ccn3* at the early phase of bone regeneration by RT–PCR, western blot, and immunofluorescence analyses. *Ccn3* transgenic mice, in which *Ccn3* expression was driven by 2.3-kb *Col1a1* promoter, showed osteopenia compared with wild-type mice, but *Ccn3* knockout mice showed no skeletal changes compared with wild-type mice. We analyzed bone regeneration process in *Ccn3* transgenic mice and *Ccn3* knockout mice by microcomputed tomography and histological analyses. Bone regeneration in *Ccn3* knockout mice was accelerated compared with that in wild-type mice. The mRNA expression levels of osteoblast-related genes (*Runx2, Sp7, Col1a1, Alpl, Bglap*) in *Ccn3* knockout mice were upregulated earlier than those in wild-type mice, as demonstrated by RT-PCR. Bone regeneration in *Ccn3* transgenic mice showed no significant changes compared with that in wild-type mice. Phosphorylation of Smad1/5 was highly upregulated at bone regeneration sites in *Ccn3* KO mice compared with wild-type mice. These results indicate that CCN3 is upregulated in the early phase of bone regeneration, and acts as a negative regulator for bone regeneration. This study may contribute to the development of new strategies for bone regeneration therapy.

ICCNS - SPRINGER AWARDS

CCN1 IS A TARGET GENE OF B-CATENIN SIGNALING AND PLAY AN IMPORTANT ROLE IN THE PROGRESSION OF LIVER CIRRHOTIC AND HEPATOCELLULAR CARCINOMA

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CCN1, a member of the CCN family of multifunctional proteins, is also found over-expressed in many types of tumor and plays dramatically different roles in tumorigenesis. Abnormal activation of the canonical Wnt signaling pathway has been implicated in carcinogenesis. Transcription of Wnt target genes is regulated by nuclear β-catenin, whose over-expression is observed in Hepatocellular Carcinoma (HCC) tissue. Firstly, we investigated the relationship between CCN1 and β-catenin in HCC. We found that while CCN1 protein was not expressed at a detectable level in the liver tissue of healthy individuals, its expression level was elevated in the HCC and HCC adjacent tissues. Over-expression of CCN1 was positively correlated with increased levels of β-catenin in human HCC samples. Activation of β-catenin signaling elevated the mRNA level of CCN1 in HepG2 cells, and inhibition of β-catenin signaling reduced both mRNA and protein levels of CCN1. We identified two TCF4-binding elements in the promoter region of human CCN1 gene and demonstrated that β-catenin/TCF4 complex specifically bound to the CCN1 promoter *in vivo* and directly regulated its promoter activity. Also, we found that over-expression of CCN1 in HepG2 cells promoted the progression of HCC xenografts in SCID mice. These findings indicate that CCN1 is a direct target of β-catenin signaling in HCC and may play an important role in the progression of HCC. Furthermore, we found that CCN1 protein was expressed in liver cirrhosis and cirrhotic tissues adjacent to hepatocellular carcinoma. During the progression of liver fibrosis mouse model, the expression tendency of CCN1 was closely associated with the severity of fibrosis. This discovery indicates that CCN1 may involve in development of liver fibrosis-hepatocellular carcinoma axis. Hepatic stellate cells (HSCs) are the main ECM-producing cells during liver fibrosis. Activated HSCs stimulates the proliferation, growth and migration of hepatocarcinoma cells *in vitro* and *in vivo*. In addition, several integrin subunits are located on HSCs membrane. So, we presume that CCN1 may involve in the progression of liver fibrosis–hepatic cirrhosis–hepatocellular carcinoma axis through regulating the activation of HSCs. We plan to demonstrate that CCN1 promotes the fibrogenic activation of HSCs through their direct binding to integrin subunit, and CCN1 enhances the founction of HSCs in driving progression of hepatocellular carcinoma in vitro and in vivo.

HIGH LEVELS OF CONNECTIVE TISSUE GROWTH FACTOR/CCN2 CAN ACCELERATE DISEASE IN A MODEL OF ACUTE LYMPHOBLASTIC LEUKAEMIA

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Acute lymphoblastic leukaemia (ALL) is the most common form of cancer in children. Despite major improvements in cure rates, a significant number of patients relapse and their prognosis remains dismal. ALL originates in the bone marrow and cell-cell interactions in this microenvironment can alter disease progression and treatment efficacy.
Connective tissue growth factor (*CTGF/CCN2*) is expressed at significantly higher levels in approximately 75 % of pre-B ALL specimens compared to normal cells. CCN2 is a secreted protein with functions in mesenchymal stem cell differentiation, fibrosis and cancer. Mechanisms of action include neo-vascularisation, migration, and proliferation. The role of CCN2 in ALL is currently unknown. Addition of CCN2 to two bone marrow stromal cell lines enhanced their proliferation rate while it had no effect on the proliferation of four pre-B ALL cell lines. Using lentiviral technology we modified a patient-derived pre-B ALL cell line (PER-371) to express and secrete high levels of CCN2, which did not alter their proliferation rate *in vitro*. However, when xenografted in NOD/SCID mice, high *CCN2*-expressing PER-371 cells showed accelerated leukaemic development. The median survival was 70 days, compared to 89 days ($p=0.03$) for mice injected with PER-371 control cells that express basal levels of *CCN2*. We determined whether high gene expression led to distinct cell homing in xenografted mice. Leukaemic cell infiltration was measured in haemopoietic organs of mouse cohorts at three time points during disease development. There were no significant differences in leukaemic cell infiltration early in disease, however, high *CCN2*-expressing PER-371 cells were significantly increased in the bone marrow approximately 2 weeks before full development of disease compared to control PER-371 xenografts (44 % vs 8 %; $p=0.01$). This suggests that high levels of *CCN2* in these cells does not influence homing, but confers a growth advantage within the bone marrow niche. Using lentiviral shRNA technology, we recently generated pre-B ALL cell lines with reduced levels of secreted CCN2, resulting in significantly reduced proliferation *in vitro* in two pre-B ALL cell lines. PER-371-shCCN2 proliferation was reduced by 99 % ($p<0.005$) and PER-377-shCCN2 by 95 % ($p<0.005$) compared to respective controls. *In vivo* studies using these cell lines are in progress and will determine if CCN2 is an effective target for treatment of ALL patients.

CCN2 IS PRESENT IN EXOSOMES FROM ACTIVATED HEPATIC STELLATE CELLS

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Introduction: Fibrotic conditions affect millions of people world-wide and cause significant morbidity and mortality. Fibrogenic pathways in the liver are principally regulated by hepatic stellate cells (HSC) which produce and respond to fibrotic mediators such as CCN2. Although the CCN2 primary translation product contains a signal peptide to direct secretion, CCN2 is a matricellular protein that appears to act in a highly localized manner by virtue of its matrix-associating properties. Here we describe a novel mode of release from HSC by which CCN2 mRNA and protein are exported from the cells in membraneous nanovesicles, or "exosomes". The recognition that CCN2 mRNA and protein are exosomal cargo molecules suggests that they exert at least some of their biological effects by exosomal shuttling between neighboring cells. **Methods:** HSC were isolated from normal Swiss Webster mice. Cells were placed in culture to induce their autonomous activation into myofibroblastic cells which were then grown in vitro for up to 11 passages. Exosomes were isolated from serum-depleted conditioned

medium by differential ultracentrifugation and characterized by Western blot, real time PCR, dynamic light scattering, zeta potential analysis, and electron microscopy. Some cells were transfected with a green fluorescent protein-CCN2 (GFP-CCN2) plasmid to permit tracking of CCN2 in exosomes and its delivery to target cells. **Results:** Exosomes from HSC were negatively charged bi-membrane vesicles, 50–150 nm in diameter, and positive for the murine exosome marker CD9. HSC-derived exosomes contained CCN2 mRNA as assessed by RT-PCR and 20-38-kDa forms of CCN2 protein as assessed by Western blot. Exosomal CCN2 mRNA levels were enhanced by transfection of the cells with GFP-CCN2 for 48 h. Addition of exosomes from GFP-CCN2-transfected donor cells to recipient Day 2 HSC cultures resulted in the detection of GFP, CCN2 and α-SMA protein and GFP transcript in the recipient cells at 24 h. **Conclusions:** CCN2 mRNA or protein is packaged by activated HSC into secreted nano-sized exosomes. Exosomal CCN2 loading is influenced by the level of CCN2 production in the cells themselves. The ability to deliver CCN2 to quiescent HSC via exosomes from activated cells suggests that the manifestation of CCN2-dependent fibrogenic pathways in HSC is influenced by exosomal cell-cell communication. We propose that *in vivo*, exosomes protect CCN2 protein and/or mRNA, allowing them to traverse the extracellular environment and circumvent either hostile conditions or sequestration in extracellular matrix. Studies to show biological actions of exosomal CCN2 in recipient cells are underway.

SHORT ORAL PRESENTATIONS OF POSTERS

FUNCTIONAL ANALYSIS OF CCN4/WISP-1 IN CHONDROGENIC DIFFERENTIATION

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Purpose: CCN4/WISP-1 (CCN4) is expressed in developing bone and is up-regulated during fracture repair. Our previous study showed that CCN4 regulates osteogenic differentiation. However, it is still unclear whether CCN4 is involved in chondrogenic differentiation. Therefore, the aim of this study was to investigate the role of CCN4 in chondrogenic differentiation.
Methods: Histological analysis was performed using E18.5 embryonic tibia from CCN4 knock-out (KO) and wild-type (WT) mice. Total RNA samples were collected from articular cartilage of CCN4 KO and WT adult mice (6–7 weeks old), and mRNA expression levels of *Sox-9*, aggrecan (*Acan*) and type II collagen (*Col2*) were analyzed by real time RT-PCR. To confirm the CCN4 function *in vitro*, gain- and loss of function analyses were performed by transfection of adenovirus expressing CCN4 (adCCN4) and small interfering RNAs (siCCN4) into human bone marrow stromal cells (hBMSCs), respectively. The chondrogenic effect of CCN4 was investigated by micromass cultures of hBMSCs. To investigate the effects of CCN4 on TGF-β signaling pathway, we examined the phosphorylation of SMAD2/3 in BMSCs.
Results: Safranin O staining data of embryonic tibia showed delayed cartilage development in CCN4 KO mice, which suggested a reduced cartilage metabolism. In fact, mRNA expression levels of *Sox-9*, *Acan* and *Col2* were significantly decreased in the cartilage of adult KO mice, compared with WT mice. *In vitro* study showed that overexpression of CCN4 significantly enhanced mRNA expression levels of *SOX-9* in hBMSCs. Although phosphorylation of SMAD2 was not induced by transduction of adCCN4 alone, TGF-β3-induced phosphorylation of SMAD2 was strongly enhanced by transduction of adCCN4. On the other hand, treatment of siCCN4 inhibited phosphorylation of SMAD2 induced by TGF-β3.
Conclusion: CCN4 regulates chondrogenic differentiation by controlling the TGF-β3-induced SMAD signaling pathway.

THE NOVEL ROLE OF CCN2 AS A REGERATORY FACTOR IN RANK/RANKL/OPG SYSTEM

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CCN2 is a multi-functional factor for connective tissue cells such as chondrocytes, fibroblasts, osteoblasts, and endothelial cells. Because we and other groups have shown that CCN2 binds to several growth factors and their receptors, this multi-functionality might be due to direct molecular interactions with other cytokines and receptors. To search additional factors binding to CCN2, we made use of a phage-display system and found receptor activator of NF-kappa B (RANK) as a possible binding partner. RANK is a member of TNF receptor family and also known as TRANCE receptor. This receptor RANK is expressed on osteoclast precursors and RANK/RANK ligand (RANKL) signaling has a critical role in osteoclastogenesis. To confirm direct binding between CCN2 and RANK, we carried out solid-phase binding assay and found specific binding of CCN2 to RANK. In addition, surface plasmon resonance (SPR) revealed interaction between CCN2 and RANK with sufficient affinity. We also found that CCN2 enhanced the RANK-mediated signaling such as NF-kappa B, ERK and JNK pathways in pre-osteoclast cell RAW264.7, whereas CCN2 did not influence to RANK-RANK ligand (RANKL) binding. These findings indicate that CCN2 directly binds to RANK and enhances RANK/RANKL signaling.
Osteoprotegerin (OPG) is known to be a decoy receptor binding to RANKL instead of RANK. We also examined the interaction of CCN2 with OPG and found that the affinity of CCN2 binding to OPG was comparable to that of RANKL to OPG. OPG markedly inhibited the binding of CCN2 to RANK, while CCN2 cancelled the inhibitory effect of OPG on osteoclast differentiation. These data shows that CCN2 also bind to OPG and contribute to osteoclast differentiation by suppressing the inhibitory effect of OPG.
In conclusion, these findings all together strongly suggest that CCN2 is the fourth factor in RANK/RANKL/OPG system, which regulates function of OPG and RANK via its direct interaction.