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The Wnt Antagonist Wif-1 Interacts With CTGF and Inhibits CTGF Activity

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Wnt inhibitory factor 1 (Wif-1) is a secreted antagonist of Wnt signalling. We recently demonstrated that this molecule is expressed predominantly in superficial layers of epiphyseal cartilage but also in bone and tendon. Moreover, we showed that Wif-1 is capable of binding to several cartilage-related Wnt ligands and interferes with Wnt3a-dependent Wnt signalling in chondrogenic cells. Here we provide evidence that the biological function of Wif-1 may not be confined to the modulation of Wnt signalling but appears to include the regulation of other signalling pathways. Thus, we show that Wif-1 physically binds to connective tissue growth factor (CTGF/CCN2) in vitro, predominantly by interaction with the C-terminal cysteine knot domain of CTGF. In vivo such an interaction appears also likely since the expression patterns of these two secreted proteins overlap in peripheral zones of epiphyseal cartilage. In chondrocytes CTGF has been shown to induce the expression of cartilage matrix genes such as *aggrecan* (*Acan*) and *collagen2a1* (*Col2a1*). In this study we demonstrate that Wif-1 is capable to interfere with CTGF-dependent induction of *Acan* and *Col2a1* gene expression in primary murine chondrocytes. Conversely, CTGF does not interfere with Wif-1-dependent inhibition of Wnt signalling. These results indicate that Wif-1 may be a multifunctional modulator of signalling pathways in the cartilage compartment.

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Wnt signalling pathways control skeletal development in a specific, either positive or negative manner (DeLise et al., 2000; Hartmann, 2006). Processes regulated by Wnt signals include differentiation and maturation of chondrocytes, joint formation and osteogenesis (for review see: Church et al., 2002; Church and Francis-West, 2002; Hartmann, 2002, 2006).

To ensure correct timing and spacing of Wnt effects, tight regulation of Wnt signalling is indispensable. Secreted antagonists of Wnt signalling, such as members of the Dickkopf (Dkk) and secreted frizzled-related protein (sFRP) families have been shown to contribute to the control of Wnt activity in skeletal development and homeostasis (Mukhopadhyay et al., 2001; Church and Francis-West, 2002; Gaur et al., 2006; Diarra et al., 2007). Wnt inhibitory factor 1 (Wif-1) is a new type of secreted Wnt antagonist, which is structurally unrelated to sFRPs or Dkk. We and others have shown that Wif-1 exerts its antagonistic activity by binding to Wnt ligands, thereby abrogating Wnt signalling (Hsieh et al., 1999; Hunter et al., 2004; Surmann-Schmitt et al., 2009a). *Wif-1* is predominantly expressed in the peripheral chondrocyte layers of epiphyseal and articular cartilage. It is capable to interfere with Wnt3a-mediated signalling in chondrogenic cells; for example, it releases Wnt3a-dependent blockade of chondrogenesis (Surmann-Schmitt et al., 2009a). Targeted disruption of the *Wif-1* gene, however, does not obviously affect normal development (own unpublished data; Kansara et al., 2009).

Interestingly, gain- and loss-of-function mutations of *shifted*, the *Wif-1* orthologue in Drosophila, do not lead to Wnt signalling-related phenotypes. In fact, the phenotypes observed demonstrate that Shifted rather affects Hedgehog than Wnt signalling. While evidence for a direct interaction of Shifted and Hedgehog has not been provided, Glise et al. have demonstrated that Shifted affects extracellular Hedgehog distribution by facilitating the movement of Hedgehog in the extracellular compartment (Glise et al., 2005).

Most signalling pathways are interlinked to signalling networks, allowing cross-talks between different pathways. For instance, cross-talks between Wnt and BMP signalling pathways during differentiation of mesenchymal progenitor cells are well established (Fischer et al., 2002; Nakashima et al., 2005). Signalling cross-talks have also been demonstrated for the Wnt pathways with TGFβ and FGF signalling (Tuli et al., 2003; Attisano and Labbe, 2004; Katoh and Katoh, 2006).

Proposing that the biological function of Wif-1 may not be confined to the regulation of Wnt signals, we used a yeast-two-hybrid (Y2H) approach to identify new protein interaction

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partners of Wif-1 that are potentially involved in other regulatory pathways. After screening a Y2H library from human HCS2/8 chondrosarcoma cells using Wif-1 as bait, one of the most abundant potential interaction partners of Wif-1 was connective tissue growth factor (CTGF, CCN2). CTGF was discovered in 1991 while screening a cDNA expression library from human vein endothelial cells (HUVEC) (Bradham et al., 1991). It is a member of the CCN family of secreted proteins and consists of four conserved domains: an insulin-like growth factor-binding protein (IGFBP) domain, a von Willebrand factor type C (VWC) domain, a thrombospondin type I (TSP) repeat and a C-terminal cysteine knot (CT) domain (Bork, 1993). The gene for CTGF is expressed in many types of cells including endothelial cells, fibroblasts, and leukocytes, but most substantially in osteoblasts and chondrocytes. CTGF has been described as a mitogenic and angiogenic factor, as a regulator of cell adhesion and migration, and as an inducer of extracellular matrix synthesis (Igarashi et al., 1993; Babic et al., 1999; Friedrichsen et al., 2003; Safadi et al., 2003; Leask and Abraham, 2006).

In this study we provide evidence for a direct interaction of Wif-1 with CTGF, which is primarily mediated by the CT domain of CTGF. Moreover, our data show that expression domains of these two genes overlap in epiphyseal and articular cartilage, which indicates that the secreted Wif-1 and CTGF proteins are present in the same regions of the extracellular compartment, possibly enabling their interaction in vivo. Finally, we demonstrate that Wif-1 is capable to block CTGF-dependent induction of *aggrecan* (*Acan*) and *collagen2a1* (*Col2a1*) gene expression, indicating that Wif-1 may interfere with CTGF activity and thus can be considered as a secreted CTGF antagonist. In the reciprocal experiment, however, CTGF did not interfere with Wif-1-mediated abrogation of canonical Wnt signalling, which may be due to the lower affinity of Wif-1 to CTGF ($K_D = 3 \times 10^{-7}$ M) than to Wnt3a ($K_D = 4 \times 10^{-9}$ M). In conclusion, this study implies that Wif-1 is not only an antagonist of Wnt signalling but may also act as a modulator of CTGF activity in the control of cartilage gene expression.

Materials and Methods

Yeast two hybrid (Y2H) screening

For the identification of protein interaction partners of WIF-1 a human *WIF-1* cDNA fragment (coding for amino acids 28-379 but lacking the intrinsic signal peptide) was amplified by PCR and cloned into the Y2H bait vector pGBKT7, resulting in the expression of a WIF-1/GAL4 binding domain fusion protein. Screening of a cDNA expression library in pGADT7 (containing the GAL4 transactivation domain) from human HCS-2/8 chondrosarcoma cells was performed as previously reported (Hattori et al., 2006, 2008; Hoshijima et al., 2006). Briefly, bait and library vectors were transformed into yeast AH109 cells, and the cells were screened on selection medium [SD/−Ade/−His/−Leu/−Trp (synthetic dropout medium lacking Ade, His, Leu and Trp)]. Positive clones were picked, and the extracted plasmid DNA was retransformed into *E. coli* prior to sequence analysis. For re-evaluation of WIF-1/CTGF interaction in yeast, AH109 cells were co-transformed with full length and truncated versions of CTGF in pGADT7 and Wif-1 in pGBKT7, and growth of transformants on selection medium [SD/−Ade/−His/−Leu/−Trp] was monitored. Generation of CTGF cDNA fragments by PCR was described earlier (Hoshijima et al., 2006; Aoyama et al., 2009).

Generation of recombinant proteins

Recombinant FLAG-His-tagged Wif-1 protein was generated by episomal expression in HEK293EBNA cells as previously reported (Surmann-Schmitt et al., 2009a). Similarly, CTGF and CTGF fragments were recombinantly expressed. Human *CTGF* cDNA comprising nucleotides 284–1,256 (GenBank NM_001901) coding for full-length CTGF without the N-terminal signal peptide was introduced into a modified pCEP-Pu vector (Wuttke et al., 2001). This vector contains a BM-40 signal peptide followed by his/myc tags and a cleavage site for enterokinase allowing the expression of N-terminally his-myc-tagged CTGF. Likewise, the cDNA coding for TSP and CT domains (nucleotides 822–1,331), and CT domain (nt 1,025–1,331) of murine *CTGF* (GenBank NM_010217) were cloned into a modified pCEP-Pu vector. IGFBP domain (nt 274–587), VWC domain (nt 587–820), and TSP domain (nt 822–1,023) were cloned in a similar way, however, with C-terminal fusion of myc/his tags.

For protein production HEK293EBNA cells were transfected with the respective expression constructs and selected with puromycin at 0.5 μg/ml. Serum-free conditioned medium was concentrated by ultrafiltration on a YM10 Amicon membrane (Millipore, Schwalbach, Germany) and purified by affinity chromatography on nickel-nitrilotriacetic acid agarose (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Size and purity of recombinant proteins were determined by SDS-PAGE.

Immunoprecipitation and Western blotting

For co-immunoprecipitation experiments 1 μg recombinant FLAG/His-tagged Wif-1 was precipitated with 1 μg mouse anti-FLAG antibody (Sigma-Aldrich, Munich, Germany, F1804) in the presence of 0.5 μg recombinant myc/His-tagged CTGF or BSA overnight at 4°C in 0.5 ml IP buffer (20 mM Tris, 25 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 1 mM DTT, pH 8.0). Similarly, recombinant murine myc/His-tagged CTGF fragments (1 μg) were precipitated with 0.5 μg rabbit anti-myc antibody (Sigma-Aldrich, C3956) in the presence of 1 μg Wif-1. Immunecomplexes were bound to protein A (rabbit anti-myc antibody) or protein G (mouse anti-FLAG antibody) and pulled down by centrifugation. After extensive washing in IP buffer bead-immunecomplexes were solubilised in Laemmli buffer and submitted to SDS-PAGE and Western blotting using mouse anti-FLAG or rabbit anti-myc antibodies, as previously described (Surmann-Schmitt et al., 2009a).

Solid phase binding assays

Protein–protein interaction studies by ELISA were performed as described previously (Aumailley et al., 1989). Briefly, solid phase was established by coating ELISA plates with 50 μl protein solution (10 μg/ml Wif-1 TBS) at 4°C overnight. After blocking with 1% BSA/TBS for 1 h at room temperature, plates were washed with TBS-T (0.05% Tween 20 in TBS). Subsequently, plates were incubated with various dilutions of soluble ligand (his/myc-CTGF) or derivatives starting from 0.5 μM in 1% BSA/TBS) for 2 h at room temperature. After extensive washing with TBS-T bound ligand was detected with either anti-CTGF or anti-myc antibody followed by HRP-conjugated goat anti-rabbit IgG antibody. HRP activity was detected with 5-amino salicylic acid and measured in an ELISA-reader at 490 nm.

Surface plasmon resonance analysis (Biacore)

Binding of CTGF to Wif-1 was analysed by surface plasmon resonance using a Biacore 2000 instrument (GE healthcare, Munich, Germany). Wif-1 was immobilised on a CM5 sensor chip by amine coupling according to manufacturer's instruction. The amount of immobilised Wif-1 was in the range of 5,000–7,000 resonance units (RU). Binding studies were performed in 0.15 M NaCl, 0.01 M HEPES (pH 7.5) containing 0.005% surfactant P20 and the chip was regenerated by 0.01 M HCl. The K_D values were calculated using the BIA evaluation software version 3.0 as previously reported (von der Mark et al., 2007).

Cell culture

Primary murine rib chondrocytes were isolated as described earlier (Surmann-Schmitt et al., 2008). Isolated chondrocytes were cultured in DMEM with 10% FCS for 16h prior to stimulation with indicated doses of human recombinant CTGF (Biomol GmbH, Hamburg, Germany) and Wif-1 in serum-free medium for 24h. HEK293T cells, 4H4 and 4C3 cells (two subclones of the MC615 murine chondrocyte cell line) were cultivated as previously described in DMEM/Ham's F12 in the presence of 10% FCS (Surmann-Schmitt et al., 2009a,b). Stimulation of these cells with CTGF, Wif-1 and Wnt3a conditioned medium was performed as indicated in medium containing 10% FCS.

Immunofluorescence

Intracellular distribution of β-catenin in 4C3 cells was determined by immunofluorescence using an anti-β-catenin antibody (H-102 Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:300, as previously reported (Surmann-Schmitt et al., 2009a). Briefly, treated cells were fixed with 4% paraformaldehyde and permeabilised in methanol. After blocking in 5% BSA cells were incubated with anti-β-catenin antibody overnight at 4°C. For detection of bound primary antibody cells were incubated with Fluorolink Cy3-labeled goat α-rabbit IgG (GE Healthcare, Munich, Germany) at a dilution of 1:800. Fluorescence was detected with a UV microscope (Carl Zeiss MicroImaging, Inc., Jena, Germany) or a Leica DMRE confocal laser microscope.

Transfection and reporter assays

One day before transfection 1.5×10^5 HEK293T cells per well were seeded in 24-well plates. Transfection was performed using 0.5 μg DNA and 6 μl PEI solution (25 kDa linear polyethylenimine: 7.5 mM monomer, pH 7.3) per well as reported by Reed et al. (2006). Canonical Wnt signalling was analysed using the TOPglow *Photinus* luciferase reporter vector (Millipore, Schwalbach, Germany) and the *Renilla* luciferase control vector phRL-SV40 (Promega GmbH, Mannheim, Germany) for normalisation. One day after transfection with TOPglow and phRL-SV40, cells were stimulated with Wnt3a-conditioned or control-conditioned medium (100 μl/ml, respectively) and indicated doses of Wif-1 and CTGF (Surmann-Schmitt et al., 2009a). After 24 h, cells were lysed in 100 μl passive lysis buffer (Promega) per well according to the manufacturer's instructions. Dual luciferase reporter assays were performed according to a protocol by Hampf and Gossen (2006). Briefly, *Photinus* and *Renilla* luciferase activities in 5 μl cell lysate were successively measured in a plate luminometer at room temperature for 10 sec, respectively. For determination of *Photinus* and *Renilla* luciferase activities 100 μl solution P/Rluc-A (200 mM Tris–HCl, 15 mM $MgSO_4$, 0.1 mM EDTA, 25 mM dithiothreitol, 1 mM ATP, 0.2 mM coenzyme A, and 200 μM luciferin, pH 8.0) and 100 μl solution P/Rluc-B (25 mM $Na4PP_i$, 10 mM NaAc, 15 mM EDTA, 500 mM Na_2SO_4, 500 mM NaCl, 50 μM APMBT [Cat No. A7507,0010; AppliChem GmbH, Darmstadt, Germany], and 4 μM benzyl-coelenterazine, pH 5.0) were injected into the reaction, respectively. *Photinus* luciferase values were normalised against the respective *Renilla* luciferase values. Means and s.d. of triplicate reactions were plotted.

Expression analyses

RNA in situ hybridisation (ISH) on paraffin sections of mouse tissues with digoxigenin-labelled antisense riboprobes for *Wif-1* and *CTGF* was carried out as reported before (Schmidl et al., 2006; Surmann-Schmitt et al., 2009a). Specific cDNA fragments for *CTGF* antisense riboprobes including nucleotides nt 1,269–1,715 of the murine *CTGF* mRNA sequence (GenBank NM_010217) were obtained by RT-PCR and introduced into the pSC-A-amp/kan cloning vector (Stratagene).

For quantitative expression analyses total RNA was prepared from stimulated cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Subsequently, mRNA was reverse transcribed using the Superscript II reverse transcriptase system (Invitrogen, Darmstadt, Germany) and quantitative determination of mRNA expression was performed by real-time RT-PCR as previously reported (Surmann-Schmitt et al., 2008). For normalisation mRNA levels of *cyclophilinA* were determined. For primer sequences please refer to (Surmann-Schmitt et al., 2009b).

Results

CTGF binds to Wif-1 in yeast

We recently demonstrated that Wif-1 interacts with a subset of cartilage-related Wnt ligands (Surmann-Schmitt et al., 2009a). In order to identify further protein interaction partners of Wif-1 we performed a yeast two hybrid screening using human WIF-1 as bait for a human HCS2/8 chondrosarcoma library in AH109 yeast cells. Plasmid DNA from resulting yeast clones grown on selection plates was isolated and transformed into *E. coli*. Subsequently, plasmid DNA was isolated from *E. coli* and gene identity was determined by sequencing. Thereby 55 clones were successfully characterised. Specific binding of these clones to Wif-1 in yeast was confirmed by retransformation of the Wif-1 bait vector and the respective library clone into AH109 yeast cells, followed by growth on selection plates. Among the characterised 55 clones, 7 carried the complete coding sequence for CTGF or 5' truncated derivatives of CTGF (Fig. 1). The 5' borders of these *CTGF* clones corresponded to nucleotide positions 32, 40 (including the full open reading frame), 497 (comprising VWC, TSP and CT domains), 737 (including TSP and CT domains) and 986 (containing only the largest part of the CT domain) of the published human *CTGF* mRNA sequence (GenBank accession: NM_001901). These results provide evidence for frequent interaction of CTGF with Wif-1 in yeast cells. Moreover, the identification of a Y2H clone containing only the CT domain indicates that this domain is sufficient for interaction with Wif-1 in yeast cells.

CTGF physically interacts with Wif-1 in vitro

Since Y2H screens tend to identify false positive interaction candidates, in particular, when screening for extracellular proteins additional studies on the physical interaction of Wif-1 and CTGF were performed in vitro. Recombinant murine Wif-1 (Flag-His tagged) and human CTGF (His-myc-tagged) proteins were produced in HEK293 EBNA cells. For in vitro binding studies recombinant Wif-1 and CTGF proteins were used in co-immunoprecipitation and solid phase assays. Pulldown of Wif-1 with a mouse anti-FLAG antibody and protein G-sepharose beads co-precipitated CTGF as detected by Western blotting using a rabbit anti-myc antibody (Fig. 2A), indicating direct

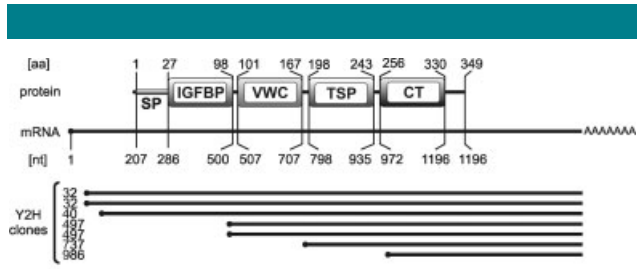

Fig. 1. CTGF-clones identified in a Y2H screen with Wif-1 and a human chondrosarcoma library contain at least the CT domain. Illustration of CTGF protein domain structure, corresponding mRNA positions and CTGF-containing Y2H clones identified in a Y2H screen.

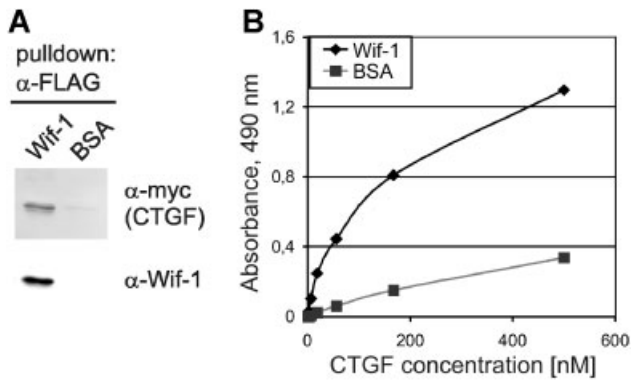

Fig. 2. Wif-1 interacts with CTGF in vitro. A: Immunoprecipitation of recombinant Wif-1-FLAG/His using α-FLAG antibody for precipitation and detection of co-precipitated CTGF-myc/His protein with an α-myc antibody by Western blotting. BSA control confirms specificity of Wif-1/CTGF binding. B: Specific and dose-dependant interaction of Wif-1 and CTGF demonstrated in a solid phase assay. Recombinant Wif-1 or BSA was coated to an ELISA plate and incubated with indicated doses of recombinant CTGF-myc/His. After washing bound CTGF was detected by an α-CTGF antibody and quantified densitometrically at 490 nm.

interaction of Wif-1 and CTGF under these conditions. This result was confirmed by solid phase assays, using Wif-1 as an immobilised ligand (Fig. 2B).

CTGF binds to Wif-1 predominantly via its C-terminal cysteine knot domain

CTGF consists of multiple domains, each responsible for interaction with a specific set of ligands. In order to identify the Wif-1-binding domain of CTGF, a series of CTGF deletion constructs was cloned into the yeast-two-hybrid vector pGADT7 and co-transformed into AH109 yeast cells together with the Wif-1 bait construct. Transformed yeast cells were seeded on selection agar plates and growth of resistant AH109 transformants was monitored after four days. The results shown in Figure 3A indicate that for interaction in yeast cells with Wif-1 a minimal CTGF construct must contain at least the von Willebrand type C domain (VWC) or the C-terminal cysteine knot domain (CT). In contrast, yeast cells transformed with constructs containing only the insulin-like growth factor binding protein domain (IGFBP) or the thrombospondin type 1 repeat (TSP) did not grow on selection plates, indicating that these domains are not capable to interact with Wif-1. In vitro similar results were obtained by co-immunoprecipitation and solid phase assays. Thus, co-immunoprecipitation experiments of recombinant myc-tagged CTGF fragments and Wif-1 indicated that the IGFBP or the TSP domain alone were not capable to significantly bind to Wif-1. In contrast, Wif-1 co-precipitated with full length CTGF, the VWC domain, and most substantially, with the CT domain alone. A fragment containing TSP and CT domain was also capable to bind to and pull down Wif-1. Note that the anti-mouse secondary antibody used for immunoblotting exhibited some cross-reactivity for the rabbit anti-myc antibody used for immunoprecipitation, resulting in a Western blot band above the specific Wif-1 band (Fig. 3B). Solid phase assays with immobilised Wif-1 or bovine serum albumin (BSA) as control confirmed that interaction of Wif-1 and CTGF is predominantly mediated by the CTGF-CT domain. In this type of assay the VWC domain exhibited only weak interaction with Wif-1, and Wif-1 interaction with IGFBP or TSP domains was barely detectable (Fig. 3C). In order to quantify the affinity of Wif-1 to CTGF and to compare it with the affinity of Wif-1 to Wnt3a, surface plasmon resonance assays were performed using a Biacore device. In these studies full length CTGF (Fig. 4B) and its CT domain (Fig. 4C) exhibited significant and similar affinity to Wif-1, which, however, was lower than the affinity of Wif-1 to Wnt3a (Fig. 4A): The calculated dissociation constants (K_D) for complexes with CTGF derivatives were approximately 300 nM, while the K_D for Wif-1-Wnt3a complexes was approximately 4 nM. In contrast, IGFBP, VWC, and TSP domains did not significantly bind to Wif-1 in this system. In total these results indicate that Wif-1/CTGF interaction is predominantly mediated by the CTGF-CT domain.

Wif-1 and CTGF are co-expressed at peripheral layers of murine cartilage

Expression of *Wif-1* and *CTGF* mRNA in the developing mouse skeleton was analysed by in situ hybridisation. In accordance with our earlier data, *Wif-1* mRNA expression in cartilage was confined to the uppermost layers of epiphyseal and articular cartilage during embryonic and postnatal development (Fig. 5Aa,a'; Ba,a'; Ca,a'; Da,a'; Surmann-Schmitt et al., 2009a). Peripheral chondrocytes of the epiphyses also exhibited high levels of *CTGF* mRNA (Fig. 5Ab,b'; Bb,b'; Cb,b'; Db,b'), while another major site of *CTGF* mRNA expression was located to the hypertrophic cartilage zone in accordance with previously published results (Nishida et al., 2003; Huang et al., 2010). Expression of *CTGF* in distal chondrocytes was detected in all developmental stages analysed, i.e. in E15.5 embryos, newborn (P1) and young (P6) mice. It overlapped with the expression pattern of *Wif-1* in the peripheral zones of epiphyseal and articular cartilage, supporting the concept of direct Wif-1/CTGF interactions in vivo.

Wif-1 abrogates CTGF-dependent induction of *aggrecan* and *Col2a1* expression in chondrocytes

Recently, CTGF has been described to interfere with Wnt signalling in *Xenopus* embryogenesis (Mercurio et al., 2004). Together with these data our findings of specific interaction of Wif-1 with CTGF and the proximity of their locations at the surface zone of cartilage raised the question, whether Wif-1 and CTGF might mutually affect Wnt or other CTGF-regulated signalling pathways.

In order to evaluate whether – and if so, how – the interaction of Wif-1 and CTGF affects Wnt signalling we performed TOPglow reporter assays to analyse canonical Wnt3a signalling in mammalian cells. As expected Wnt3a induced TOPglow reporter activity in HEK293T cells, indicating induction of the canonical Wnt signalling pathway. Wif-1 was able to block this activation, confirming our earlier data on this system (Surmann-Schmitt et al., 2009a). Interestingly, however, we could not observe any effect of CTGF on Wnt3a signalling nor on its repression by Wif-1 (Fig. 6A). Moreover, analyses of Wnt3a-dependent β-catenin accumulation in 4C3 chondrogenic cells did not reveal any influence of CTGF on canonical Wnt signalling or its abrogation by Wif-1 as detected by immunofluorescence (Fig. 6B,C). Together these data suggest that CTGF does not appear to modulate Wnt3a-dependent signalling or its abrogation by Wif-1 in HEK293T or murine chondrogenic cells. This implies that the interaction of CTGF and Wif-1 may have other consequences than the modulation of Wnt signalling in a chondrogenic environment in mammals.

CTGF is known to enhance expression of *aggrecan* (*Acan*) mRNA in chondrocytes (Nakanishi et al., 2000). In order to test the possibility of Wif-1 interfering with CTGF activity, we

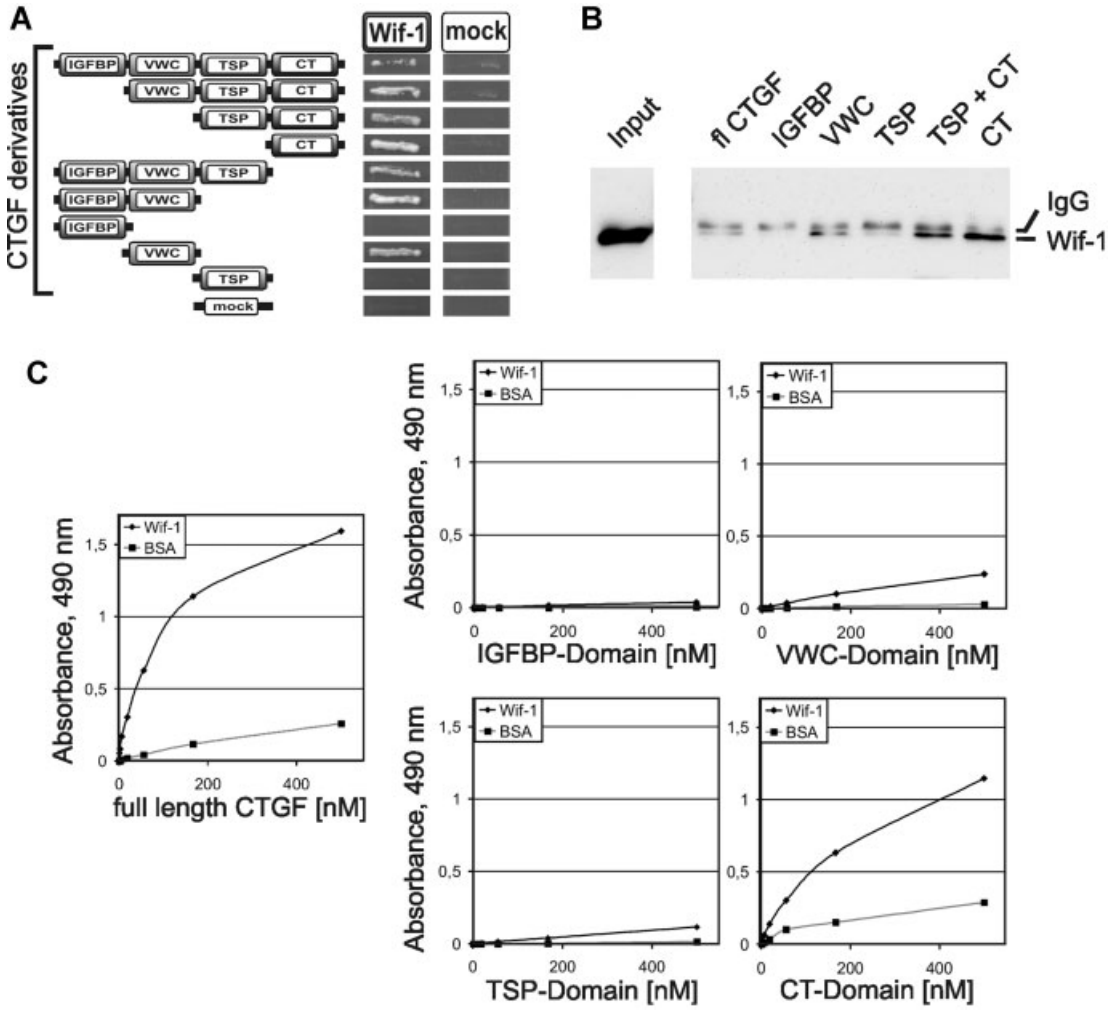

Fig. 3. Mainly the CT domain of CTGF is responsible for interaction with Wif-1. A: Y2H analysis of Wif-1 binding to truncated CTGF fragments. Yeast AH109 cells were co-transformed with a Wif-1/GAL4 binding domain fusion construct and CTGF deletion constructs fused to GAL4 transactivation domain as indicated. Yeast cells were plated on agar plates containing selection medium. Growth indicates GAL4 activity mediated by interaction of Wif-1 with the respective CTGF fragment. Mock control using a GAL4 binding domain vector without Wif-1 indicates specificity. B: Immunoprecipitation of myc-tagged recombinant CTGF fragments indicates that the CT-domain potently co-immunoprecipitates Wif-1/FLAG-His. Recombinant myc/His-tagged CTGF fragments were pulled down using an α-myc antibody in the presence of recombinant Wif-1/FLAG-His. Co-precipitated Wif-1/FLAG/His was detected using an αFLAG antibody by Western blotting. Input indicates apparent molecular weight of Wif-1/FLAG-His in the same experiment. Note that the upper bands mark the heavy chain of the antibody used for pulldown (IgG). C: Specific and dose-dependant binding of Wif-1 to full length (fl) CTGF and to the CT domain demonstrated in a solid phase assay. Recombinant Wif-1 or BSA was coated to an ELISA plate and incubated with indicated doses of the respective recombinant myc/His-tagged CTGF fragments. After washing bound CTGF fragments were detected by an α-myc antibody and quantified densitometrically at 490 nm.

treated primary murine rib chondrocytes with CTGF in the presence or absence of Wif-1 for 24h and analysed *Acan* and *Col2a1* mRNA levels by real-time RT PCR (Fig. 7A). As expected, CTGF (50 ng/ml) stimulated *Acan* expression approximately 9-fold. The addition of Wif-1 (2 μg/ml) almost completely reversed CTGF-dependent up-regulation of *Acan* expression, while Wif-1 alone did not significantly alter *Acan* mRNA levels. *Col2a1* mRNA expression was regulated in a similar fashion by CTGF and Wif-1. The same experiment was performed with the chondrogenic cell line 4H4 (Surmann-Schmitt et al., 2009b). Although the absolute enhancement of *Acan* and *Col2a1* expression by CTGF was less pronounced in these cells, again Wif-1 demonstrated an inhibitory effect of CTGF-dependent gene induction (Fig. 7B). This indicates a potential new role for Wif-1 as an inhibitor of CTGF function.

Discussion

In this study we provide several lines of evidence for direct interaction of the secreted Wnt antagonist Wif-1 and the matricellular, multidomain protein CTGF. The interaction is mediated predominantly by the CT domain of CTGF and to a lower extent by the VWC domain. We further demonstrate that the mRNA expression domains of *Wif-1* and *CTGF* overlap in peripheral layers of epiphyseal and articular cartilage, indicating that these two secreted proteins may be present in the same extracellular compartments. Finally, we demonstrate that Wif-1 impairs the *aggrecan*- and *Col2a1*-enhancing activity of CTGF in chondrocytes and therefore propose Wif-1 as a novel modulator of CTGF activity.

Data indicating that the Wif-1 orthologue Shifted affects Hedgehog signalling instead of Wnt signalling in *Drosophila*

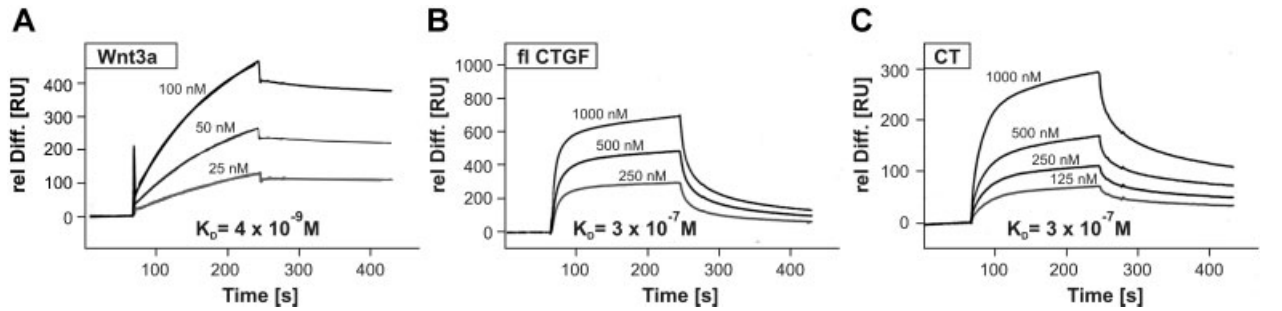

Fig. 4. Full-length CTGF and CT domain bind to Wif-1 with low affinity. For quantification of the affinities of Wif-1 to Wnt3a and CTGF, respectively, binding of Wif-1 to Wnt3a or CTGF was analyzed by surface plasmon resonance (Biacore). Wif-1 was immobilised on a sensor chip and binding of Wnt3a or CTGF (fragments) was determined as shown. The IGFBP, VWC, and TSP domains did not exhibit significant binding to Wif-1. In contrast full-length CTGF (B) and the CT domain (C) both bound to Wif-1 with a calculated K_D of app. 300 nM, while Wif-1/Wnt3a complexes (A) exhibited a K_D of app. 4 nM.

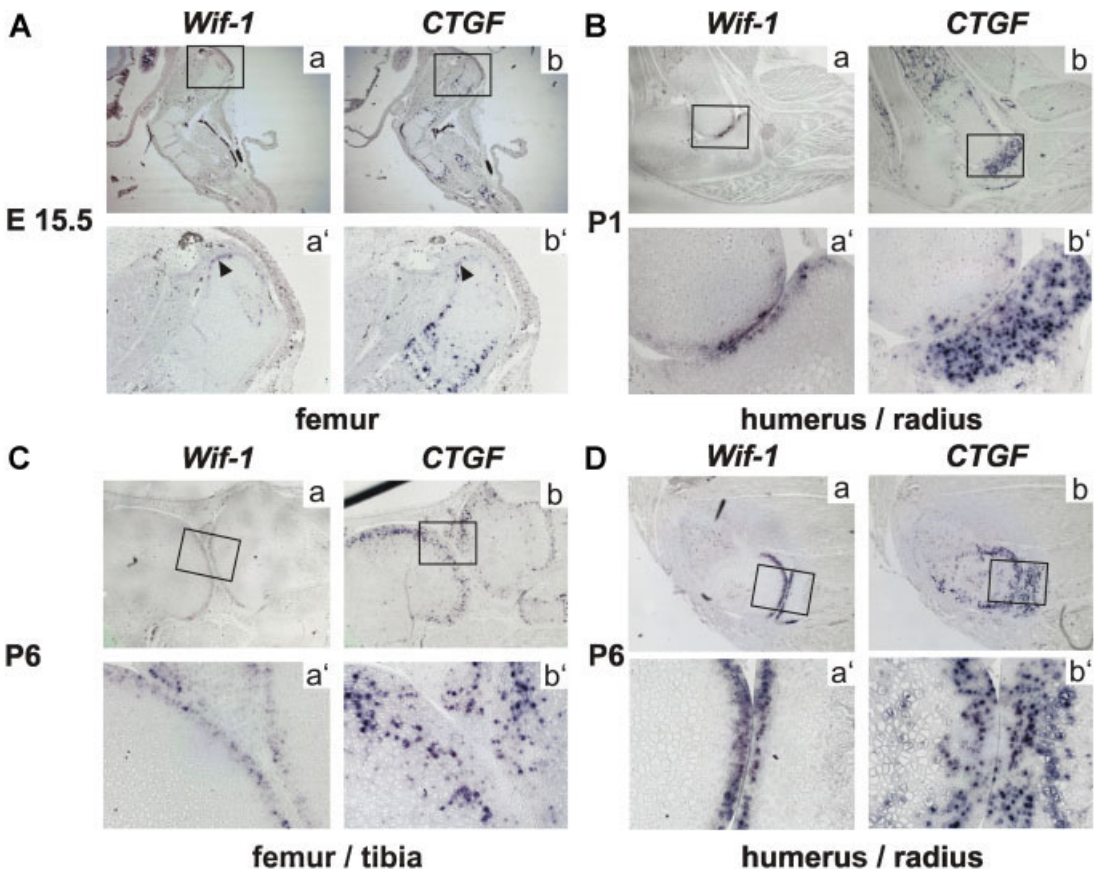

Fig. 5. The expression domains of *Wif-1* and *CTGF* overlap in peripheral layers of epiphyseal and articular cartilage. The expression patterns of *Wif-1* and *CTGF* were determined by RNA in situ hybridisation (ISH) on parallel consecutive sections of paraffin-embedded murine hind and forelimbs. A: Expression of *Wif-1* (a,a') and *CTGF* (b,b') in the hindlimb of an E15.5 mouse embryo. Co-expression of *Wif-1* and *CTGF* is observed in peripheral layers of the proximal femur epiphysis (a',b', arrow heads). B and D: At the elbow joint of newborn (B) and 6 day-old (D) mice *Wif-1* (a,a') and *CTGF* (b,b') were co-expressed at similar superficial regions of the distal epiphyseal cartilage of humerus and radius. C: Similarly, *Wif-1* (a,a') and *CTGF* (b,b') expression co-localised at peripheral layers of epiphyseal cartilage of 6 day-old mice in the knee joint. In all panels rectangles in a and b indicate the position of higher magnification panes a' and b', respectively.

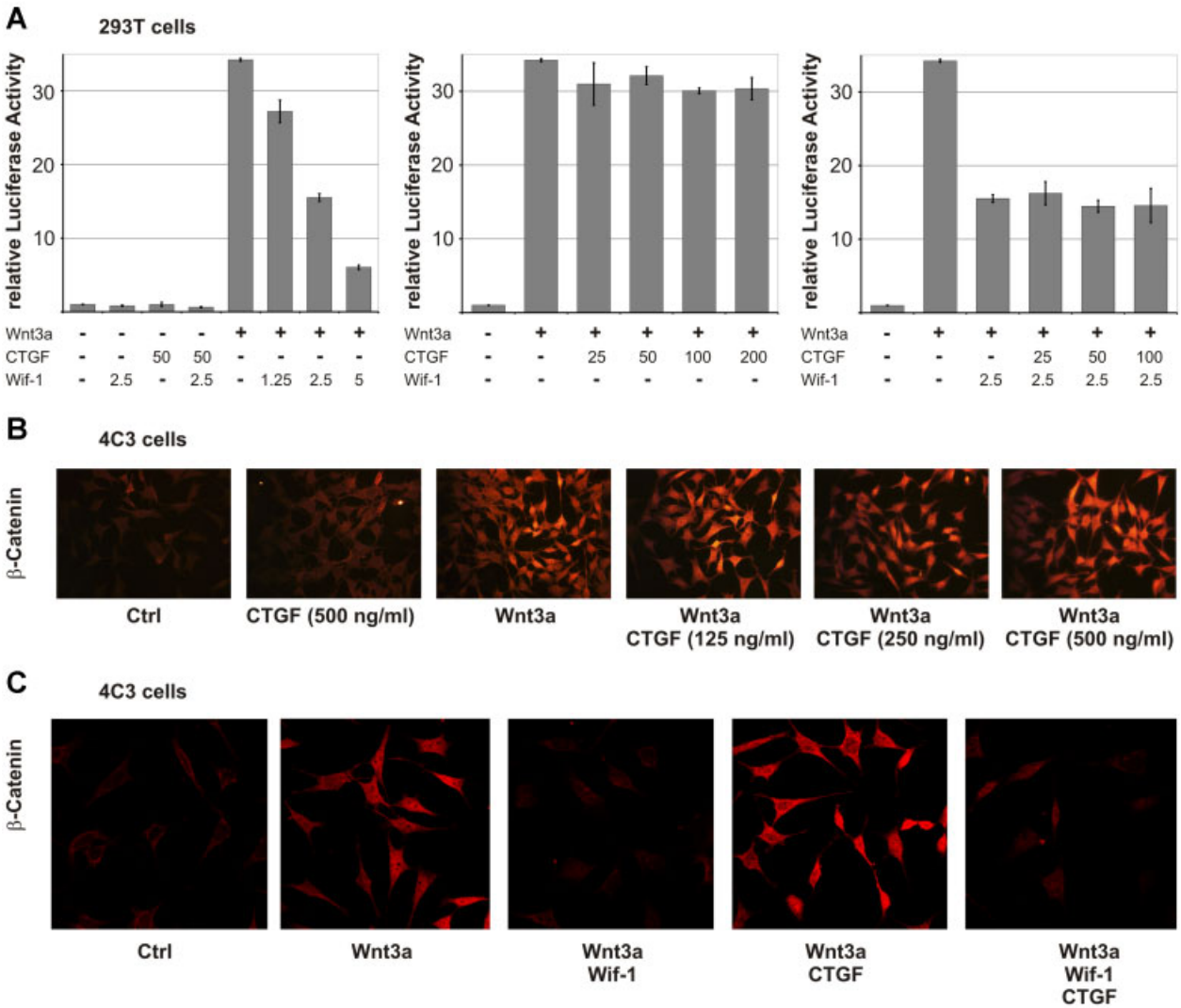

Fig. 6. CTGF does not affect Wnt3a signalling or its inhibition by Wif-1 in HEK293T cells or 4C3 chondrocytes. A: HEK293T cells transfected with a TOPglow reporter system and a *Renilla* luciferase control vector for normalisation were stimulated with the indicated doses of recombinant Wif-1 and recombinant CTGF in the presence of 100 μl/ml Wnt3a conditioned medium or control conditioned medium. Reporter assays were carried out 24 h after stimulation. Normalised TOPglow luciferase activities are shown. Means (n = 3) are plotted as bars and error bars indicate s.d. B: 4C3 cells were stimulated with 100 μl/ml Wnt3a-conditioned medium or the respective control medium and indicated doses of recombinant CTGF. Five hours after stimulation accumulation of β-catenin was detected by immunofluorescence and visualised by UV microscopy. C: 4C3 cells were treated as described in B and stimulated with Wnt3a-conditioned medium supplemented as indicated with recombinant Wif-1 (2.5 μg/ml) and recombinant CTGF (125 ng/μl). Immunofluorescence of β-catenin was detected by confocal laser microscopy.

support the hypothesis that Wif-1 might also trigger signalling other than Wnt pathways (Glise et al., 2005). Moreover, *Wif-1*-deficient mice develop a higher susceptibility to osteosarcoma (Kansara et al., 2009). Such a finding has not been described for any other knockout models for Wnt antagonists (Lories and Luyten, 2009). This may indicate that Wif-1 might have more tasks than solely the control of Wnt activities in vivo, and that its anti-tumour activity resides at least in part in such secondary functions.

In a Y2H screen for protein interaction partners of Wif-1, clones coding for CTGF were among the most frequently found. Since Y2H screens tend to identify false positive candidates for protein binding partners, in particular when screening for secreted proteins, we performed further in vitro experiments including co-immunoprecipitation assays, solid phase binding studies and surface plasmon resonance (Biacore) analyses to provide additional evidence for direct Wif-1-CTGF interactions. All these analyses confirmed specific interactions between Wif-1 and CTGF. We quantitatively determined the affinity of these two proteins by Biacore and calculated an apparent dissociation constant (K_D) of approximately 300 nM, which is significantly lower than the affinity of Wif-1-Wnt3a interaction ($K_D = 4$ nM); thus, CTGF appears to be a low-affinity ligand of Wif-1. The affinities of CTGF to other secreted molecules are also typically somewhat higher, for example for TGFβ ($K_D = 30$ nM), BMP7 ($K_D = 14$ nM) or VEGF (two classes of binding sites with app. $K_D = 26$ nM and 125 nM, respectively; Abreu et al., 2002; Inoki et al., 2002; Nguyen et al., 2008). Nevertheless, interactions with dissociation constants in the range of those observed for Wif-1-CTGF interaction have been described for other growth factors including TGFβ, which binds to its receptor TGFR II with a K_D of 150 nM, or for insulin-like

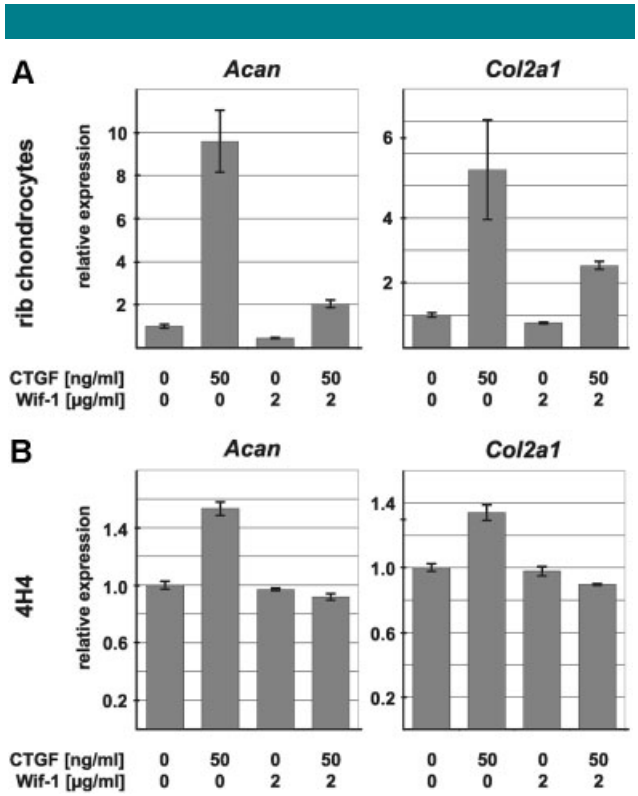

Fig. 7. Wif-1 interferes with CTGF-dependent induction of *Acan* and *Col2a1* gene expression. A: Primary murine rib chondrocytes were treated with indicated doses of recombinant CTGF and Wif-1 in serum-free medium for 24 h. Subsequently, total RNA was isolated and *Acan* and *Col2a1* mRNA expression was determined by real-time RT-PCR. B: 4H4 cells were treated with indicated doses of recombinant CTGF and Wif-1 in medium containing 10% FCS for 24h before *Acan* and *Col2a1* expression levels were determined as described in A). Cyclophilin A mRNA levels were assessed for standardisation of relative mRNA expression. Real-time PCR reactions were run in triplicates and bars indicate mean relative expression. Standard deviations are shown.

growth factor I (IGF-I) and unphosphorylated IGF binding protein 1 (IGFBP-1; $K_D = 150–700$ nM; Goetschy et al., 1996; Seferovic et al., 2009). Interestingly, CTGF has also been reported to bind to IGF with low affinity and therefore has been classified as an IGF binding protein (IGFBP-8) (Kim et al., 1997).

Although initial studies on *CTGF* expression in chondrocytes reported transcripts primarily in hypertrophic and calcifying cartilage, more recent studies provide evidence for significant *CTGF* mRNA levels also in peripheral layers of epiphyseal and articular cartilage, which is in line with the data presented here (Nakanishi et al., 1997; Friedrichsen et al., 2003; Oka et al., 2007; Huang et al., 2011). Since CTGF is known as an inducer of matrix synthesis, *CTGF* expression in peripheral layers of epiphyseal and articular cartilage may play a role in stabilising the cartilage surface. Thus, CTGF has been implicated in articular cartilage regeneration (Nishida et al., 2004). Wif-1 null mice do not exhibit abnormal cartilage formation during normal development (Kansara et al., 2009; Surmann-Schmitt et al., 2009a). However, the high Wif-1 expression at the cartilage border together with the findings that Wif-1 abrogates both, canonical Wnt signalling – which is known to inhibit the expression of cartilage matrix genes – and cartilage matrix formation inducing CTGF signalling, may indicate that Wif-1 is involved in the finetuning of cartilage matrix turnover at the cartilage surface (Nakanishi et al., 2000; Surmann-Schmitt et al., 2009a). The importance of this finetuning of catabolic and anabolic signals may, however, only become apparent under challenging conditions such as ageing or disease.

CTGF has been shown to elicit a wide range of different cellular responses. Therefore, it is plausible that the activity of this multi-purpose molecule is tightly regulated at multiple levels: First, *CTGF* mRNA expression underlies stringent control of growth factors, such as TGFβ, Endothelin-1, BMPs and Wnt factors, environmental stimuli like hypoxia, and transcription factors including Smad, Ets-1, Sp1 and Hif-1 (Luo et al., 2004; Leask and Abraham, 2006). Moreover, posttranslational events control mRNA stability (Luo et al., 2004; Leask and Abraham, 2006 for review). Proteolytic cleavage of CTGF, which is frequently observed in biologic fluids, results in smaller fragments with distinct biological activities. This process is discussed as another mechanism to regulate overall CTGF activity (Brigstock et al., 1997; Lau and Lam, 1999; Grotendorst and Duncan, 2005).

Our data indicate that Wif-1 is capable to bind to CTGF and to interfere with its biologic activity. Thus, we introduce a further level to the regulation of CTGF activity, which is mediated by a secreted antagonist. Although high doses of recombinant Wif-1 (2 µg/ml) were needed to block CTGF activity, these high doses appear to be typical for Wif-1 activity: we show here and in a previous work that similar doses (1.25–10 µg/ml) of recombinant Wif-1 are necessary for effective attenuation of Wnt3a activity (Surmann-Schmitt et al., 2009a). Moreover, another group has recently observed significant activity of commercially available recombinant Wif-1 (R&D) at similar doses, ranging from 20–50 nM (approximately 1–2.5 µg/ml; Cho et al., 2009).

The CT domain of CTGF mediates interactions with fibronectin, heparansulfate proteoglycans and integrins (Gao and Brigstock, 2004; Hoshijima et al., 2006). Through these interactions CTGF may promote cell adhesion via integrins and proteoglycans in several cell types (for review, Leask and Abraham, 2006). Mercurio et al. have shown that *Xenopus* CTGF interferes with Wnt signalling by binding with its CT domain to the Wnt receptor frizzled 8 (Fzd8) and to the co-receptor LRP6 (Mercurio et al., 2004). Yet, Rooney et al. have recently shown that CTGF induces β-catenin-dependent Wnt signalling in human mesengial cells (Rooney et al., 2011). These data raised the question, whether Wif-1 might affect these CTGF interactions. In our study, however, we demonstrate that CTGF does not affect canonical Wnt signalling in HEK293T cells or 4C3 chondrocytes. These data imply that CTGF might affect canonical Wnt signalling in different ways, depending on the cellular background.

A possible explanation for the apparent conflict between the findings that Wif-1 inhibited CTGF-induced *aggrecan* and *Col2a1* expression in chondrocytes on the one hand, while on the other hand CTGF did not interfere with Wif-1-mediated inhibition of Wnt signalling are the different affinities of Wif-1 for Wnt ligands and CTGF. In fact surface plasmon resonance analyses revealed that the affinity of Wif-1 to Wnt3a ($K_D = 4 \times 10^{-9}$ M) is higher by two orders of magnitude than its affinity to CTGF or its CT domain ($K_D = 3 \times 10^{-7}$ M, both).

Although CTGF appears to bind to Wif-1 predominantly via the CT domain, we also observed interaction between the VWC domain and Wif-1, albeit at a lower level. This is of particular interest since the VWC domain has been described to be responsible for the interaction with TGFβ, thereby enhancing TGFβ signalling (Abreu et al., 2002). TGFβ, in turn, has been shown to be an inducer of cartilage matrix production as it promotes *Acan* and *Col2a1* expression of chondrocytes (Chandrasekhar and Harvey, 1988; Morales, 1991; Qi and Scully, 1998).

As stated above, *Wif-1*-deficient mice are more susceptible to the development of osteosarcomas than wild-type animals,

while this is not observed in other Wnt antagonist null mice (Kansara et al., 2009). Considering Wif-1 as a CTGF antagonist, it is intriguing that in a mouse model for osteolytic lung cancer bone metastasis, neutralising antibodies against CTGF resulted in decreased bone metastasis and accompanying microvasculature (Shimo et al., 2006). It may be speculated that the CTGF antagonistic function of Wif-1 might contribute to the anti-tumorigenic effect of Wif-1.

Taken together our data demonstrate for the first time direct protein interaction of Wif-1 with a secreted factor other than Wnt ligands. Our study clearly demonstrates that CTGF is a major binding partner of Wif-1. Our findings furthermore demonstrate that these two factors are co-expressed at superficial cartilage layers. Finally we show that, while CTGF is not capable to affect Wif-1-dependent abrogation of Wnt signalling in chondrocytes, Wif-1 interferes with CTGF-dependent induction of *Acan* and *Col2a1* gene expression in this cell type. Thus, we introduce Wif-1 as a novel secreted modulator of CTGF activities in cartilage.

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