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RESEARCH ARTICLE

The gene expression profile induced by Wnt 3a in NIH 3T3 fibroblasts

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Abstract Wnt proteins play important roles in regulating cell differentiation, proliferation and polarity. Wnts have been proposed to play roles in tissue repair and fibrosis, yet the gene expression profile of fibroblasts exposed to Wnts has not been examined. We use Affymetrix genome-wide expression profiling to show that a 6-h treatment of fibroblasts of Wnt3a results in the induction of mRNAs encoding known Wnt targets such as the fibrogenic pro-adhesive molecule connective tissue growth factor (CTGF, CCN2). Wnt3a also induces mRNAs encoding potent pro-fibrotic proteins such as TGFβ and endothelin-1 (ET-1). Moreover, Wnt3a promotes genes associated with cell adhesion and migration, vasculature development, cell proliferation and Wnt signaling. Conversely, Wnt3a suppresses gene associated with skeletal development, matrix degradation and cell death. Results were confirmed using real-time polymerase chain reaction of cells exposed to Wnt3a and Wnt10b. These results suggest that Wnts induce genes promoting fibroblast differentiation towards angiogenesis and matrix remodeling, at the expense of skeletal development.

Keywords CCN2 · Microarray · Fibroblasts

Introduction

Wnt signaling, a conserved molecular mechanism in animals, enables cells to converse with each other to coordinate a remarkable variety of cellular processes, such as cell fate, differentiation, cell survival and migration during morphogenesis (Logan and Nusse 2004). Wnts can stimulate numerous intracellular signal transduction cascades, including the essentially linear canonical β-catenin pathway, which regulates gene expression, and non-canonical pathways, which regulate many other aspects of cell biology (Cadigan and Liu 2006). A hallmark of the canonical Wnt pathway is the stabilization and nuclear localization of β-catenin (Schohl and Fagotto 2002). Without the presence of Wnts, β-catenin is targeted for degradation, whereas the presence of Wnts results in β-catenin stabilization and nuclear localization (Willert and Jones 2006). There are several different Wnt proteins, often with different overall effects on cells (Musgrove 2004).

Wnt signaling has been shown to play an essential role in brain, limb, mammary, skin, and most recently cardiovascular and lung development (Morrisey 2003). Chilosi and colleagues (2003) observed accumulation of nuclear β-catenin, the downstream mediator of the canonical Wnt pathway, in both epithelial and mesenchymal (myofibroblast) cell lineages in adult human lung and evidence of activation of this pathway in idiopathic pulmonary fibrosis. In this light, it has been suggested that fibroblasts isolated from fibrotic lesions of scleroderma patients possess a so-called 'Wnt-signature' (Gardner et al. 2006). However, it is unclear what exactly a 'Wnt signature' is in fibroblasts, nor if application of Wnts to fibroblasts result in the direct activation a fibrogenic or tissue repair program in fibroblasts. Wnts, in particular Wnt 3a, is however known to induce the expression of members of the CCN family of proteins [including CTGF (CCN2) and cyr61 (CCN1)] in mesenchymal cells (Luo et al. 2004; Si et al. 2006; Leask and Abraham 2006). CCN proteins play key roles in matrix remodeling, tissue repair and fibrogenesis (Leask and

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Table 1 Cluster analysis ($p<0.02$) of mRNAs (out of 145 total) induced more than twofold in fibroblasts by Wnt3a treatment (6 h)

Affymetrix ID	Increase	Gene name	
Cell cycle			
1416268_at	2.266	Ets2	E26 avian leukemia oncogene 2
1422631_at	7.433	Ahr	Aryl-hydrocarbon receptor
1424325_at	2.098	Esco1	Establishment of cohesion 1 homolog 1
1423250_a_at	3.321	Tgfb2	TGFbeta2
1427267_at	2.098	Zfp469	Zinc finger protein 469
1429658_a_at	2.15	Smc2	Structural maintenance of chromosomes 2
1439766_x_at	3.715	Vegfc	Vascular endothelial growth factor C
1440739_at	3.275	Vegfc	Vascular endothelial growth factor C
1449027_at	2.54	Rhou	Ras homolog gene family, member U
1449028_at	3.193	Rhou	Ras homolog gene family, member U
1450922_a_at	3.483	Tgfb2	TGFbeta2
1450923_at	3.086	Tgfb2	TGFbeta2
1452459_at	2.837	Aspm	Abnormal spindle-like, microcephaly associated
1453851_a_at	4.925	Gadd45g	Growth arrest and DNA-damage-inducible 45 gamma
Angiogenesis			
1416039_x_at	3.067	CCN1(Cyr61)	Cysteine-rich protein 61
1419417_at	3.372	Vegfc	Vascular endothelial growth factor C
1423250_a_at	3.321	Tgfb2	TGFbeta2
1434025_at	2.145	Klf5	Kruppel-like factor 5
1434776_at	2.154	Sema5a	Semaphorin 5A
1438133_a_at	3.229	CCN1(Cyr61)	Cysteine rich protein 61
1439766_x_at	3.715	Vegfc	Vascular endothelial growth factor C
1440739_at	3.275	Vegfc	Vascular endothelial growth factor C
1442340_x_at	3.186	CCN1(Cyr61)	Cyr61
1450922_a_at	3.483	Tgfb2	TGFbeta2
1450923_at	3.086	Tgfb2	TGFbeta2
1451021_a_at	2.227	Klf5	Kruppel-like factor 5
1451924_a_at	2.113	Edn1	Endothelin 1
1457823_at	2.908	Cyr61	Cyr61
Cytoskeleton			
1417011_at	2.017	Sdc2	Syndecan 2
1425060_s_at	3.794	Rsn	Restin
1428579_at	2.38	Fmnl2	Formin-like 2
1431098_at	2.668	Clip1	CAP-GLY domain containing linker protein 1
1435551_at	5.084	Fhod3	Formin homology 2 domain containing 3
1437554_at	2.382	Plec1	Plectin 1
1450650_at	2.356	Myo10	Myosin X
1455056_at	2.645	Lmo7	LIM domain only 7
Cell motility/adhesion			
1418733_at	2.436	Twist1	Twist gene homolog 1
1423250_a_at	3.321	Tgfb2	TGFbeta2
1426300_at	2.559	Alcam	Activated leukocyte cell adhesion molecule
1426301_at	2.572	Alcam	Activated leukocyte cell adhesion molecule
1427267_at	2.098	Zfp469	Zinc finger protein 469
1437466_at	2.865	Alcam	Activated leukocyte cell adhesion molecule
1434776_at	2.154	Sema5a	Semaphorin 5A
1437467_at	2.846	Alcam	Activated leukocyte cell adhesion molecule
1450922_a_at	3.483	Tgfb2	TGFbeta1
1450923_at	3.086	Tgfb2	TGFbeta2
1454966_at	2.161	Itga8	Integrin alpha 8
Wnt signaling			
1428808_at	2.016	Prickle2	Prickle-like 2
1450044_at	2.687	Fzd7	Frizzled homolog 7
1450772_at	2.195	Wnt11	Wingless-related MMTV integration site 11
1459804_at	2.105	Crebbp	CREB binding protein

Table 1 (continued)

Affymetrix ID	Increase	Gene name	
1459804_at	2.105	Crebbp	CREB binding protein
1460729_at	2.606	Rock1	Rho-associated coiled-coil containing protein kinase 1
CCN family			
1438133_a_at	3.229	CCN1(Cyr61)	Cysteine rich protein 61
1442340_x_at	3.186	CCN1(Cyr61)	Cysteine rich protein 61
1457823_at	2.908	CCN1(Cyr61)	Cysteine rich protein 61
1416953_at	2.42	CCN2 (Ctgf)	Connective tissue growth factor
1448593_at	2.232	CCN4(Wisp1)	WNT1 inducible signaling pathway protein 1
1419015_at	3.661	CCN5(Wisp2)	WNT1 inducible signaling pathway protein 2
1448594_at	3.023	CCN4(Wisp1)	WNT1 inducible signaling pathway protein 1

Abraham 2006). Although these results suggest that Wnts play key roles in initiating tissue remodeling and fibrotic expression profiles in fibroblasts, this hypothesis has not been tested. In fact, the overall effect of Wnts on fibroblast gene expression is wholly unknown.

In this report, we use Affymetrix gene profiling and real-time polymerase chain reaction (PCR) analysis to identify the transcripts induced by Wnts in NIH 3T3 fibroblasts. Our results provide new insights into the role that Wnts may play in fibroblast differentiation.

Materials and methods

Cell cultures NIH 3T3 fibroblasts (ATCC) were cultured in low glucose Dulbecco's modified Eagle's medium (DMEM), 10% calf serum, and antibiotic/antimycotic solution (Invitrogen, Burlington, Ontario) at 37°C, 5% CO_2. Cells were serum starved in DMEM/0.5% calf serum for 18 h and treated with or without Wnt3a (R and D Systems) for additional lengths of time as indicated. Alternatively, conditioned media obtained from cell lines overexpressing Wnt 1, 4, 5 or 10 or containing empty expression vector (courtesy Cun-Yu Wang, University of Michigan) was added to DMEM/0.5% calf serum in a 1:1 ratio. RNA was then harvested using Trizol (Invitrogen) and quantified as previously described (Shi-wen et al. 2004, 2006; Kennedy et al. 2007).

RNA quality assessment, probe preparation and gene chip hybridization and analysis

Microarrays and analysis were performed essentially as previously described (Shi-wen et al. 2004; Liu et al. 2007; Kennedy et al. 2007). All Gene Chips were processed at the London Regional Genomics Centre (Robarts Research Institute, London, ON; http://www.lrgc.ca). RNA was harvested (Trizol, Invitrogen), quantified and quality (RNA degradation and DNA contamination) was assessed using the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA) and the RNA 6000 Nano kit (Caliper Life Sciences, Mountain View, CA). Quality data was then analyzed using the Degradometer (http://www.dnaarrays.org). Biotinylated complimentary RNA (cRNA) was prepared from 10 μg of total RNA as per the Affymetrix GeneChip Technical Analysis Manual (Affymetrix, Santa Clara, CA). Double-stranded cDNA was synthesized using SuperScript II (Invitrogen) and oligo(dT) 24 primers. Biotin-labeled cRNA was prepared by cDNA in vitro transcription using the Bizarre High-Yield RNA Transcript Labeling kit (Enzo Brioche, New York, NY) incorporating biotinylated UTP and CTP. Fifteen μg of labeled cRNA was hybridized to Mouse Genome 430 2.0 Gene Chips for 16 h at 45°C as described in the Affymetrix Technical Analysis Manual (Affymetrix, Santa Clara, CA). Gene Chips were stained with streptavidin–phycoerythrin, followed by an antibody solution and a second streptavidin–phycoerythrin solution, with all liquid handling performed by a GeneChip Fluidics Station 450 (Affymetrix). Gene Chips were scanned with the Affymetrix GeneChip Scanner 3000 (Affymetrix). Signal intensities for genes were generated using GCOS1.2 (Affymetrix) using default values for the Statistical Expression algorithm parameters and a target signal of 150 for all probe sets and a normalization value of 1. Normalization was performed in GeneSpring 7.2 (Agilent Technologies Inc.). The RMA preprocessor was used to import data from the.cel files. Data were first transformed, (measurements less than 0.01 set to 0.01) and then normalized per chip to the 50th percentile, and per gene to wild-type control samples. Experiments were performed twice, and fold changes were identified using the GeneSpring filter. Data presented in Table 1 are an average of these independent studies. The fold change between treated and untreated samples had to be at least twofold to identify a transcript as being altered. These criteria had to be met in both sets of experiments. A list of the two fold changes was compiled and exported into EASE for further analysis. These group-

Table 2 Cluster analysis ($p<0.02$) of mRNAs (out of 159 total) decreased more than twofold in fibroblasts by Wnt3a treatment (6 h)

Affymetrix ID	Fold decrease	Gene name	
Skeletal development			
1418175_at	0.365	Nr1i1	Vitamin D receptor
1418176_at	0.27	Nr1i1	Vitamin D receptor
1421073_a_at	0.386	EP4	Prostaglandin E receptor 4 (subtype EP4)
1421979_at	0.33	HPDR	Phosphate regulating gene (hypophosphatemia)
1423500_a_at	0.33	Sox5	SRY-box containing gene 5
1424950_at	0.206	sox9	SRY-box containing gene 9
1427277_at	0.16	Kitl	Transcribed locus
1439753_x_at	0.412	Six4	Sine oculis-related homeobox 4 homolog
1440827_x_at	0.442	Sox5	SRY-box containing gene 5
1451538_at	0.437	Sox9	SRY-box containing gene 9
1456862_at	0.415	Six4	Sine oculis-related homeobox 4 homolog
Endopeptidase activity			
1417109_at	0.444	Tinagl	Tubulointerstitial nephritis antigen-like
1422561_at	0.457	Adamts5	Aggrecanase-2
1433434_at	0.468	AW551984	Expressed sequence AW551984
1437506_at	0.458	Adamts6	A disintegrin-like and metallopeptidase with thrombospondin type 1 motif, 6
1435697_a_at	0.455	Mmp12	Matrix metalloprotenase 12
1450716_at	0.426	Adamts1	A disintegrin-like and metallopeptidase with thrombospondin type 1 motif, 1
1450658_at	0.416	Adamts5	Aggrecanase-2
1418981_at	0.44	Casp12	Caspase 12
1421979_at	0.33	Phex	Phosphate regulating gene (hypophosphatemia)
Programmed cell death			
1415832_at	0.417	Agtr2	Angiotensin II receptor, type 2
1417130_s_at	0.452	Angptl4	Angiopoietin-like 4
1418981_at	0.44	Casp12	Caspase 12
1424950_at	0.206	Sox9	SRY-box containing gene 9
1426752_at	0.477	Phf17	PHD finger protein 17
1434601_at	0.498	Amigo2	Adhesion molecule with Ig like domain 2
1442884_at	0.349	Hgf	Hepatocyte growth factor
1449297_at	0.446	Casp12	Caspase 12
1449033_at	0.397	Tnfrsf11b	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)
1451538_at	0.437	Sox9	SRY-box containing gene 9
1451866_a_at	0.445	Hgf	Hepatocyte growth factor
1452179_at	0.423	Phf17	PHD finger protein 17
1452985_at	0.452	Uaca	Uveal autoantigen with coiled-coil domains and ankyrin repeats
Transcription			
1418175_at	0.365	Nr1i1	Vitamin D receptor
1418176_at	0.27	Nr1i1	Vitamin D receptor
1422607_at	0.425	Etv1	Ets variant gene 1
1423233_at	0.388	Cebpd	CCAAT/enhancer binding protein (C/EBP), delta
1427277_at	0.16	Kitl	Kit1
1434736_at	0.416	Hlf	Hepatic leukemia factor
1435828_at	0.332	Maf	Musculoaponeurotic fibrosarcoma (v-maf)
1456862_at	0.415	Six4	Sine oculis-related homeobox 4 homolog
1439753_x_at	0.412	Six4	Sine oculis-related homeobox 4 homolog
1447849_s_at	0.339	Maf	Musculoaponeurotic fibrosarcoma (v-maf)
1450684_at	0.356	Etv1	Ets variant gene 1
1456060_at	0.301	Maf	Musculoaponeurotic fibrosarcoma (v-maf)
1416302_at	0.388	Ebf1	Early B-cell factor 1
1416301_a_at	0.493	Ebf1	Early B-cell factor 1
1439789_at	0.481	Ebf1	Early B-cell factor 1
1428349_s_at	0.432	Ebf3	Early B-cell factor 3
1440827_x_at	0.442	Sox5	SRY-box containing gene 5
1451538_at	0.437	Sox9	SRY-box containing gene 9

Table 2 (continued)

Affymetrix ID	Fold decrease	Gene name	
1423500_a_at	0.33	Sox5	SRY-box containing gene 5
1424950_at	0.206	Sox9	SRY-box containing gene 9
G protein coupled receptor signaling			
1415832_at	0.417	Agtr2	Angiotensin II receptor, type 2
1417625_s_at	0.282	Cxcr7	Chemokine (C-X-C motif) receptor 7
1420349_at	0.137	Ptgfr	Prostaglandin F receptor
1421073_a_at	0.386	Ptger4	Prostaglandin E receptor 4 (subtype EP4)
1421470_at	0.395	Grpr	Gastrin releasing peptide receptor
1425814_a_at	0.223	Calcrl	Calcitonin receptor-like
1440777_x_at	0.334	Ptgfr	Prostaglandin F receptor
1449828_at	0.375	Ptgfr	Prostaglandin F receptor
1453924_a_at	0.16	Ptgfr	Prostaglandin F receptor
1456858_at	0.385		G protein-coupled receptor 149

ings were restricted to the GO Biological Process and subjected to Fisher exact probability and Benjamini as the primary statistics and multiplicity correction respectively. These groupings had to have at least six members/functional groupings that were part of the GO Biological Process system ($p<0.02$) to be considered relevant. Note that the list of CCN family members was compiled independent of the cluster analysis. The relevant lists were exported back into the affymetrix.com website to compile the gene name and functions.

Real time RT-PCR RNA was harvested using the Qiashredder and RNeasy kit (Qiagen) and used for real-time RT-PCR. Twenty-five nanograms of RNA was reverse transcribed and

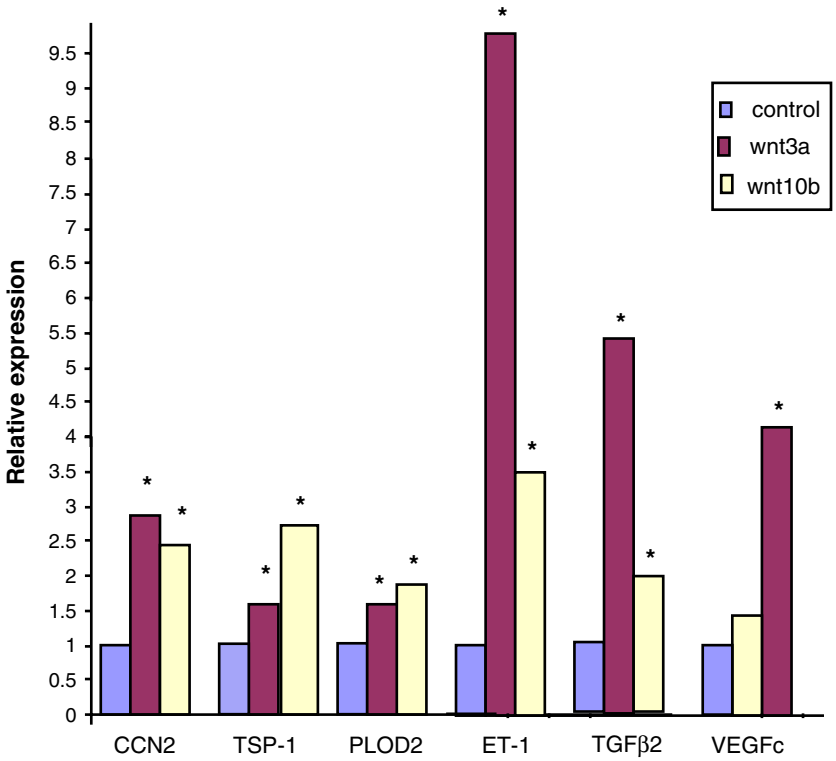

Fig. 1 Wnts 3a and 10b induce gene expression in fibroblasts. Wnt3a induces mRNA expression in fibroblasts. NIH 3T3 fibroblasts were treated with or without Wnt3a (150 ng/ml, 6 h). RNAs were harvested, and subjected to real time PCR analysis to detect 18S rRNA and RNAs as indicated. Experiments were performed thrice. A representative experiment is shown (three replicates). Variation within samples was less than 10%. NIH 3T3 fibroblasts were also treated with or without conditioned media prepared from cells stably transfected with empty expression vector or vector encoding Wnt10b. RNAs were harvested, and subjected to real time PCR analysis to detect 18S rRNA and RNAs as indicated. Untreated or vector-alone-treated samples are expressed as 1 (control). Experiments were performed at least twice. A representative experiment is shown (three replicates). CCN2, TSP-1 (thrombospondin-1), VEGF-C, PLOD2, TGFβ2 and ET-1 transcripts were examined. Variation within samples was less than 10%. Significance is indicated by an *asterisk*

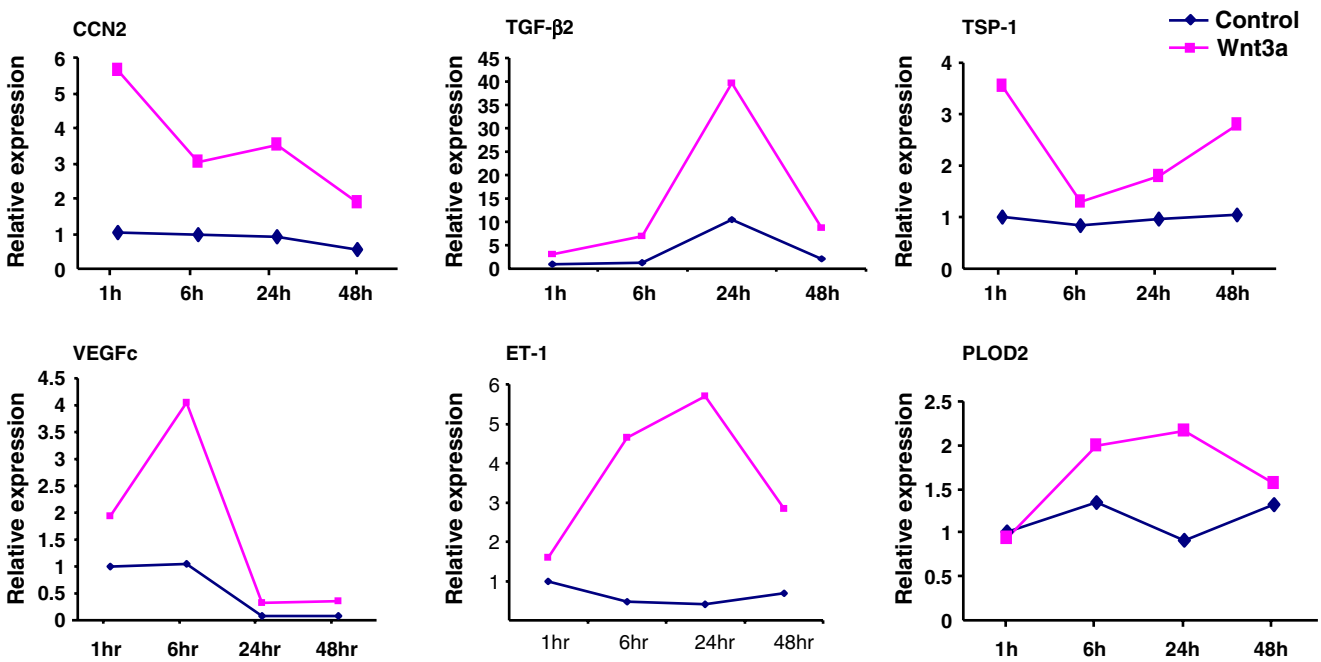

Fig. 2 Wnt 3a induces gene expression in fibroblasts. Time-course analysis. NIH 3T3 fibroblasts were treated with or without Wnt3a (150 ng/ml) for the time periods as indicated. RNAs were harvested, and subjected to real time PCR analysis to detect 18S rRNA and target RNAs as indicated. Experiments were performed twice. A representative experiment is shown (three replicates). CCN2, TSP-1 (thrombospondin-1), VEGF-C, PLOD2, TGFβ2 and ET-1 transcripts were examined. Variation within samples was less than 10%

amplified using TaqMan Assays on Demand (Applied Biosystems) in a 15-μl reaction containing primers (Assays on Demand, Applied Biosystems) and 6-carboxyfluroscein labeled TaqMan MGB probe. Reverse transcriptase qPCR Mastermix (Applied Biosystems) was added to samples and the ABI Prism 7900 HT sequence detector (Perkin–Elmer–Cetus, Vaudreuil, QC) was used according to manufacturer's instructions to detect amplified sequences. Samples were run in triplicate, transcripts were measured in picograms and expression values were standardized to control values from 18S rRNA primers. Statistical analysis was done using one way ANOVA and Tukey's post hoc test on GraphPad.

Transfection assays For transfection assays, cells were plated at a density of 25,000 cells/well in a 24 well plate. Cells were allowed to grow for 24 h at 37°C, 5% CO_2. Cells were then transfected with polyfect (Qiagen) as described by the manufacturer. Cells were transfected with plasmids containing a CCN2 promoter fused to a secreted alkaline phosphatase (SEAP) reporter gene. Cells were transfected with 1 μg of CCN2-SEAP constructs that contained the −805 to +17 region of the CCN2 promoter (Abraham et al. 2000; Holmes et al 2001). Cells were cultured for 16 h in serum-free media then treated with or without wnts for 24 h. To control for transfection efficiency cells were cotransfected with 0.5 μg of a cytomegalovirus (CMV) promoter–β-galactosidase (β-gal) reporter gene (Clontech, Palo Alto, CA, USA) construct. Promoter assays were performed with a Phospha-Light kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's protocol and SEAP reporter expression was adjusted for differences in β-galactosidase expression as determined by a Galacto-Star kit (Applied Biosystems) according to manufacturer's protocol. Data was expressed as average values ± standard deviation of at least three replicates and at least two independent trials. Measurement of SEAP levels

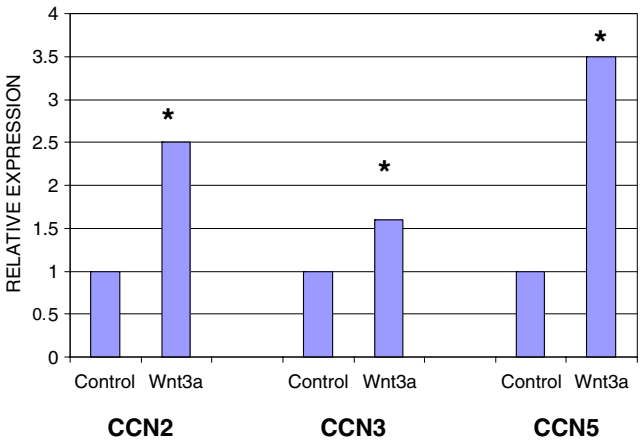

Fig. 3 Wnt 3a induces CCN2, CCN5 and CCN3 mRNAs. NIH 3T3 fibroblasts were treated with or without Wnt3a (150 ng/ml) for the time periods as indicated. RNAs were harvested, and subjected to real time PCR analysis to detect 18S rRNA and target RNAs as indicated. Experiments were performed twice. A representative experiment is shown (three replicates). CCN2, CCN5 and CCN3 transcripts were examined. Note that Wnt3a was less effective at inducing CCN3 mRNA than at inducing CCN2 and CCN5 mRNAs. Variation within samples was less than 10%

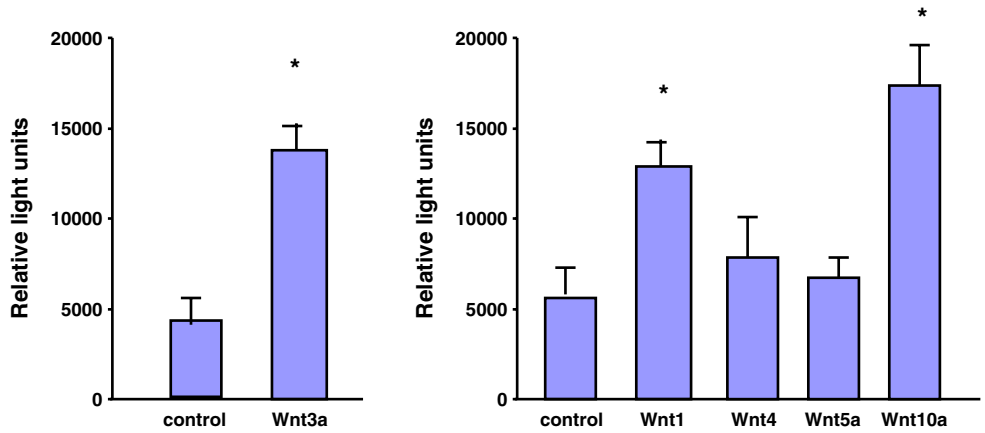

Fig. 4 Wnts 1, 3a and 10b, but not Wnts 4 or 5a, induce the CCN2 promoter in fibroblasts. NIH 3T3 fibroblasts were transfected with a CCN2 promoter/SEAP reporter construct, which contains the region spanning from −805 to + 17 of the CCN2 promoter (Abraham et al. 2000). After a serum starvation step, cells were treated with or without Wnt3a (150 ng/ml, 24 h) or with or without conditioned media from cells stably transfected with empty expression vector or expression vector encoding Wnt 1, 4, 5a or 10b (24 h). Experiments were performed thrice. A representative experiment is shown (N=6, average ± standard deviation is shown). Significance is indicated by an *asterisk*

were obtained from an LMax II 384 luminometer (Molecular Devices, Sunnyvale CA, USA) and SoftMax Pro 4.7.1 (Molecular Devices, Sunnyvale CA, USA). Levels were measured in relative light units and were standardized to control values from β-gal. Statistical tests were done using one-way ANOVA and Tukey's post hoc test on GraphPad.

Results

mRNAs whose expression are altered by exposure of NIH 3T3 fibroblasts to Wnt3a

To determine the effect of Wnts on fibroblasts, NIH 3T3 fibroblasts were incubated in serum-free media for 24 h and exposed to Wnt3a (150 ng/ml) for 6 hours. RNAs were extracted and subjected to Affymetrix genome-wide profiling. There were 145 genes induced greater that twofold by Wnt3a. Cluster analysis revealed that genes induced upon Wnt treatment included those mediating angiogenesis, Wnt signaling, cell adhesion and motility, cell proliferation and the cytoskeleton (Table 1). These mRNAs include CCN1 and CCN2, as anticipated, but also those encoding potent pro-differentiation/angiogenic proteins such as TGFβ2, endothelin-1 (ET-1) and vascular endothelial growth factor, all of which are involved with tissue remodeling and repair (Leask and Abraham 2006; Shi-wen et al. 2004, 2006; Tonnesen et al. 2000). Conversely, exposure of NIH 3T3 fibroblasts to Wnt3a reduced expression of 159 mRNAs including those mediating skeletal development, cell death and endopeptidase (matrix metalloproteinase) activity (Table 2). These results suggest that Wnt exposure is promoting angiogenesis at the expense of bone formation, and matrix production and remodeling at the expense of matrix degradation.

To further investigate the role of Wnt3a in the induction of gene expression in fibroblasts, we verified our gene expression profiling data using real-time PCR. We showed that a 6 h treatment of fibroblasts with Wnt3a resulted in an induction of CCN2, ET-1, TGFβ2 and VEGF-C mRNAs (Fig. 1). Based on these data, we also investigated whether Wnt3a could also induce additional genes involved with tissue remodeling and repair such as PLOD2 and thrombospondin-1 (van der Slot et al. 2003; Murphy-Ullrich and Poczatek 2000). To extend our results to other members of the Wnt family, we exposed fibroblasts to Wnt10b for 6 hours. Wnt 10 b induced a similar set of mRNAs to Wnt3a, although Wnt10b was not able to significantly induce VEGF-C (Fig. 1). To further analyze the ability of Wnts to induce gene expression in fibroblasts, fibroblasts were exposed in the presence or absence of Wnt3a for up to 48 h. RNA was prepared and subjected to real-time PCR analysis using appropriate primers. These mRNAs were induced with slightly differing kinetics, but were nonetheless sustained over a 48 h period (Fig. 2).

Wnt3a induces CCN2 (CTGF) and CCN5 (Wisp2) and CCN3 (nov) mRNA expression in NIH 3T3 fibroblasts

Our microarray analysis revealed, as expected, that CCN family members were induced upon Wnt3a treatment (Table 1). Of this family, CCN1, CCN2, CCN4 and CCN5 but not CCN3 (nov) mRNAs were detected as being increased upon Wnt3a treatment (Table 1). Real time PCR analysis confirmed that CCN2 and CCN5 were potently

induced by Wnt3a, conversely CCN3 (nov) was induced approximately 50% (Fig. 3). These results confirm our microarray studies and indicate that CCN3 is not a potent responder to Wnt3a in fibroblasts.

Wnts 1, 3a and 10b, but not Wnt 4 and 5a, induce the CCN2 promoter in NIH 3T3 fibroblasts

Our Affymetrix gene profiling and real-time PCR analysis confirmed previous results that Wnt3a induced CCN2 mRNA and protein in mesenchymal cells (Luo et al. 2004). For our studies, we considered that CCN2 was a key target of Wnt signaling because of the role of CCN2 as a key marker and mediator of wound healing and fibrogenesis (Blom et al. 2002; Leask and Abraham 2006). Accordingly, to obtain insights into the mechanism underlying Wnt action on fibroblasts, we investigated whether (a) the canonical Wnts (Wnt3a and 10b) could induce the CCN2 promoter. To perform this analysis, we transfected NIH 3T3 fibroblasts with a CCN2 promoter/reporter construct bearing nucleotides −805 and +17 of the promoter (Abraham et al. 2000; Leask et al. 2003) and exposed these cells to Wnts 3b and 10a. Wnts induced the CCN2 promoter (Fig. 4). To further assess the mechanisms by which Wnts act on the CCN2 promoter, we addressed whether additional Wnts could induce the CCN2 promoter. We found that an additional canonical Wnt, Wnt1, induced the CCN2 promoter (Fig. 4). Conversely, non-canonical Wnts 4 and 5a (Kuhl et al. 2000) were not able to induce the CCN2 promoter. These data extend the notion that Wnts regulate the CCN family, and in particular CCN2, in fibroblasts.

Discussion

Previous studies have found that Wnts are induced during tissue remodeling and repair (Okuse et al. 2005). However, the direct effect of Wnts on gene expression in fibroblasts has not been ascertained until this report. Wnt addition caused the induction of known Wnt targets such as members of the CCN family (Luo et al. 2004; Si et al. 2006). That Wnts induced CCN1, CCN2, CCN4 and CCN5 are not surprising as these are known Wnt targets (Luo et al. 2004; Si et al. 2006; Leask and Abraham 2006). Canonical, but not non-canonical, Wnts activate the CCN2 promoter; the mechanism underlying this activation is beyond the scope of our current paper. However, it in interesting that Wnt3a only modestly induced CCN3 (nov) mRNA. In this regard, CCN3 was recently shown to inhibit Wnt signaling (Rydziel et al. 2007) and thus that the lower magnitude of CCN3 induction by Wnt3a may be expected.

Fibrotic fibroblasts have been considered to have a ‘Wnt signature’ as revealed by cluster analysis of Affymetrix gene profiling data (Gardner et al. 2006). However, Wnt 3a did not cause an as potent induction of other profibrotic/matrix remodeling genes as, say, application of TGFβ or ET-1 to fibroblasts (Shi-wen et al. 2004, Liu et al. 2007; Leask and Abraham 2004). For example, α-smooth muscle actin (α-SMA) was not detected in our studies. However, in our current report we found that Wnt3a also induced mRNA expression of TGFβ2 and ET-1 (and indeed CCN2). We are currently evaluating if the Wnt cascade can induce excessive tissue remodeling/repair or fibrogenesis through these intermediaries.

It is interesting to note that Wnts enhanced the expression of angiogenenic and matrix remodeling genes at the expense of genes involved with skeletal development and in particular chondrogenesis (e.g. sox9). Previous evidence has suggested that Wnts induce an osteogenic lineage (in fact via CCN 1; Baron et al. 2006; Si et al. 2006). However, prior to initiating the osteogenic lineage, chondrogenesis is required, for which the sox9 is essential (Akiyama et al. 2005). Our results are therefore in good agreement with recent data that physical and functional interactions exist between β-catenin and Sox9. In vivo, either overexpression of *Sox9* or inactivation of β-*catenin* in chondrocytes of mouse embryos produces a similar phenotype of dwarfism with decreased chondrocyte proliferation, delayed hypertrophic chondrocyte differentiation, and endochondral bone formation (Akiyama et al. 2004). Moreover, Sox9 inhibits activation of β-catenin-dependent promoters and stimulates degradation of β-catenin (Akiyama et al. 2004), suggesting that chondrogenesis is controlled by interactions between Sox9 and the Wnt/β-catenin signaling pathway. Moreover, it is also intriguing that Wnt3a induced pro-mitotic mRNAs yet suppressed pro-apoptotic mRNAs. These results are consistent with recent observations that Wnt3a promotes proliferation of mesenchymal stem cells (Baksh and Tuan 2007). Collectively, our data support the notion that Wnts are potent modulators of fibroblast differentiation.

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