Wnt 10b activates the CCN2 promoter in NIH 3T3 fibroblasts through the Smad response element

Shaoqiong Chen · Andrew Leask

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Abstract Wnt proteins elevate expression of the CCN family. For example, Wnt10b induces the fibrogenic pro-adhesive molecule connective tissue growth factor (CTGF, CCN2) in NIH 3T3 fibroblasts. Wnt10b activates the CCN2 minimal promoter. In this report, we map the Wnt10b response element in the CCN2 minimal promoter to the previously identified Smad response element. These results suggest that Wnts may cross-talk with the Smad signaling pathway to induce fibrotic responses in fibroblasts.

Keywords CTGF · CCN2 · Smad · Wnt

Introduction

Wnts (such as Wnt10b) stimulate numerous intracellular signal transduction cascades, including the canonical β -catenin pathway (Kühl et al. 2000). In the absence of Wnts, β -catenin is degraded whereas the presence of Wnts results in β -catenin stabilization and nuclear localization (Willert and Jones 2006). Wnts play an essential role in brain, limb, mammary, skin, and cardiovascular and lung development (Logan and Nusse 2004). It has been suggested that fibroblasts isolated from fibrotic lesions of scleroderma patients possess a so-called ‘Wnt-signature’ (Gardner et al. 2006). However, the application of Wnts to fibroblasts results not in the direct activation of collagen mRNA

expression, complete fibrogenic or tissue repair program, but rather is likely to do so indirectly through the induction of genes thought to influence collagen production, such as members of the CCN family of proteins [including CTGF (CCN2) and *cyr61* (CCN1)], endothelin-1 and transforming growth factor β (Chen et al. 2007).

Previously, we had shown that Wnt10b activates CCN2 expression at least in part through elements in the CCN2 promoter (Chen et al. 2007). In this report, we use promoter deletion and mutation constructs to map the Wnt response element. Our results give new insights into the role that Wnts may play in fibroblast differentiation.

Materials and methods

Cell culture 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% CO₂. Conditioned media obtained from cell lines over-expressing Wnt 10b 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, as described previously (Chen et al. 2007).

Transfection assays For transfection assays, cells were plated at a density of 25000 cells/well in a 24 well plate. Cells were allowed to grow for 24 h at 37°C, 5% CO₂. 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 μ g of CCN2-SEAP constructs that

S. Chen · A. Leask (✉)
CIHR Group in Skeletal Development and Remodeling,
Division of Oral Biology and Department of Physiology
and Pharmacology, Schulich School of Medicine and Dentistry,
Dental Sciences Building, University of Western Ontario,
London, ON, Canada N6A 5C1
e-mail: Andrew.Leask@schulich.uwo.ca

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 Wnt10b for 24 h. To control for transfection efficiency cells were cotransfected with $0.5\mu\text{g}$ 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 $\pm$ standard deviation of at least 3 replicates and at least two independent trials. Measurement of SEAP levels 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

Wnt10b induces the CCN2 promoter (Chen et al. 2007). To elucidate the elements in the CCN2 promoter mediating this response, NIH 3T3 fibroblasts were transfected with a CCN2 promoter/reporter construct bearing nucleotides -805 and $+17$ of the promoter and, confirming previous data, Wnt10b induced the CCN2 promoter (Fig. 1). We found that removal of the segment of the CCN2 promoter residing between -805 and -166 removed responsiveness to Wnt10b (Fig. 1). Residing within this element is a functional Smad recognition motif (Holmes et al. 2001). Mutation of the Smad element abolished the ability of the CCN2 promoter to respond to Wnt10b (Fig. 1). Thus Wnt10b activates the CCN2 promoter, likely through Smads.

Discussion

Canonical, but not non-canonical, Wnts activate the CCN2 promoter (Chen et al. 2007). In the case of Wnt10b, elements within the minimal -805 to $+17$ CCN2 promoter mediate this

Fig. 1 Wnt 10b induce the CCN2 promoter in fibroblasts via the Smad response element. 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). CTGF promoter mutation and deletion constructs were also employed, as indicated (Abraham et al. 2000; Holmes et al. 2001). After a serum starvation step, cells were treated with or without conditioned media from cells stably transfected with empty expression vector or expression vector encoding Wnt 10b (24 hours). Experiments were performed thrice. A representative experiment is shown ($N=6$, average $\pm$ standard deviation is shown). Fold change in response to Wnt 10b or vector alone is shown. Significance is indicated by *

induction. In this report, we map the Wnt10b response element to the previously identified Smad response element of the CCN2 promoter (Holmes et al. 2001).

It is now recognized that Wnts crosstalk with the TGF β signaling pathway (Guo and Wang 2009). For example, although the promoter of *gastrin* contains both Smad and Lef/Tcf binding sites, either site alone can recruit Smads and β -catenin, which act as mutual co-factors facilitated by the p300 co-activator protein (Lei et al. 2004). Moreover, Smad3- β -catenin cross-talk has been observed in human mesenchymal stem cells (hMSCs). In particular, TGF- β and Wnt cooperatively stimulate the proliferation of hMSCs and inhibit their differentiation into the osteocytic and adipocytic lineages, thereby supporting hMSC self-renewal (Jian et al. 2006). In addition, Smad3 and β -catenin co-regulate a cohort of genes in these cells that are otherwise not known to be TGF- β or Wnt targets, such as the Src family tyrosine kinase BLK (Jian et al. 2006). Finally, myostatin-inhibited adipogenesis in human bone marrow-derived mesenchymal stem cells are mediated, in part, by activation of Smad3 and cross-communication of the TGF β /Smad signal to Wnt/ β -catenin/TCF4 pathway, with Smad3 directly interacting with β -catenin (Guo et al. 2008). Our results showing that the Wnt10b activates the CCN2 promoter via the Smad response element are consistent with these data.

Collectively, our results emphasize that cross-talk exists between the Wnt and TGF β pathway and that this interaction may contribute to elevated gene expression in tissue repair and fibrosis.

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