RESEARCH ARTICLE

Cell type-specific regulation of CCN2 protein expression by PI3K–AKT–FoxO signaling

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Abstract The biological activity of connective tissue growth factor (CTGF, CCN2) is regulated at the level of intracellular signaling leading to gene expression, and by its extracellular interaction partners which determine the functional outcome of CCN2 action. In this overview, we summarize the data which provide evidence that one of the major signaling pathways, phosphatidylinositol-3 kinase (PI3K)–AKT signaling, shows a remarkable cell type-dependence in terms of regulation of CCN2 expression. In smooth muscle cells, fibroblasts, and epithelial cells, inhibition of this pathway either reduced CCN2 expression or was not involved in CCN2 gene expression depending on the stimulus used. In microvascular endothelial cells by contrast, activation of PI3K–AKT signaling was inversely related to CCN2 expression. Upregulation of CCN2 upon inhibition of PI3K–AKT was also observed in primary cultures of human endothelial cells (HUVEC) exposed to laminar flow in an in vitro flow-through system. In different types of endothelial cells, FoxO transcription factors, which are negatively regulated by AKT, were identified as potent activators of CCN2 gene expression. In HUVEC, we observed a correlation between enhanced nuclear localization of FoxO1 and increased synthesis of CCN2 protein in areas of non-uniform shear stress. These data indicate that FoxO proteins are key regulators of CCN2 gene expression which determine the effect of PI3K–AKT activation in terms of CCN2 regulation. *Short summary* Phosphatidylinositol-3 kinase (PI3K)–AKT signaling shows a remarkable cell type-dependence in terms of regulation of CCN2 expression. In endothelial cells activation of PI3K - AKT signaling was inversely related to CCN2 expression. FoxO transcription factors, which are negatively regulated by AKT, were identified as potent activators of CCN2 gene expression.

Keywords AKT · Connective tissue growth factor · Phosphatidylinositol-3 kinase · FoxO · Endothelial cell

Abbreviations

CCN2	Connective tissue growth factor
CTGF; HUVEC	Human umbilical vein endothelial cells
PI3K	Phosphatidylinositol-3 kinase
TGF	Transforming growth factor

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Introduction

Connective tissue growth factor (CTGF, CCN2) is a multifunctional protein, which is secreted and functions as a matricellular protein. Accordingly, the biological activity of CCN2 is regulated both at the level of intracellular signaling leading to gene expression, and by its extracellular interaction partners, which determine the functional

outcome of CCN2 action. Multiple signaling pathways have been related to CCN2 gene expression, some of which, such as transforming growth factor (TGF)beta—Smad signaling, seem to work in different cell types, or are shared by multiple stimuli, such as activation of RhoA Rho-kinase signaling (Chaqour and Goppelt-Struebe 2006; Cicha & Goppelt-Struebe 2009). Other pathways leading to CCN2 induction seem to be rather specifically detected in a restricted number of cell types, for example, activation of Rac-1 has only been related to CCN2 expression in gingival fibroblasts and chondrocytes (Black and Trackman 2008; Woods et al. 2009). In this brief overview, we will concentrate on the impact of the activation of one of the major signaling pathways, which is operative in all cells, namely phosphatidylinositol-3 kinase (PI3K)–AKT signaling on CCN2 gene expression. We will summarize the published data and present evidence for cell type-specific regulation of CCN2 by PI3K–AKT signaling.

Activation of AKT may lead to the modulation of multiple pathways, giving rise to a highly complex signaling network (e.g. (Jiang and Liu 2008; Chaqour et al. 2006)). In smooth muscle cells either exposed to mechanical stretch (Chaqour et al. 2006) or activated by serum or sphingosine-1 phosphate (Chowdhury and Chaqour 2004), PI3K–AKT signaling contributed to CCN2 promoter activation. By contrast, inhibition of PI3K did not interfere with TGFbeta-induced CTGF induction in lung fibroblasts (Xie et al. 2005). In line with these data, we did not observe a role of PI3K–AKT signaling for CCN2 expression in tubular epithelial cells stimulated with hepatocyte growth factor, which is known to activate AKT (Kroening et al. 2009). In experiments with renal fibroblasts stimulated with the microtubule disrupting agent colchicine, we observed that the response of the cells depended on the cell culture conditions: When the cells were cultured on a rigid surface, upregulation of CCN2 gene expression was independent of PI3K–AKT signaling, whereas inhibitors of PI3K significantly reduced CTGF gene expression, when the cells were cultured in collagen gels (Graness et al. 2006). This differential reactivity may be related to the link between focal adhesion kinase and PI3K, which plays a role in the survival of fibroblasts in collagen gels (Xia et al. 2004). Analyzing VEGF-dependent induction of CCN2, Suzuma et al. observed inhibition of CCN2 gene expression upon interference with AKT signaling in bovine retinal endothelial cells (Suzuma et al. 2000). However, the downstream mediators of AKT signaling were not investigated in this or in any of the other studies. In recent studies with different microvascular endothelial cell lines we demonstrated that PI3K–AKT may also negatively regulate CCN2 expression as discussed in detail below (Samarin et al. 2009). To extend these findings, we investigated the regulation of CCN2 expres-sion in primary cultures of human umbilical vein endothe-lial cells (HUVEC) cultured under flow conditions which more closely resemble the in vivo conditions compared to static culture conditions.

Methods

HUVEC were isolated from freshly delivered umbilical cords and grown on 0.1% gelatin-coated dishes as described (Muehlich et al. 2004). HUVEC were seeded in flow-through cell culture slides (Ibidi®, Munich, Germany) and exposed to steady laminar shear stress for 20 h at 10 dyne/cm^2 (Cicha et al. 2008). Bifurcating slides were used to analyze cells exposed to non-uniform shear stress. Protein expression was detected by indirect immunocyto-chemistry with the following antibodies: polyclonal goat anti-CTGF (1:100; Santa Cruz), anti-goat IgG coupled to Alexa Fluor 488 (1:500; Molecular Probes), polyclonal rabbit anti-FoxO1 (1:100, Cell Signaling) and anti-rabbit IgG coupled to Alexa Fluor 555 (1:500, Molecular Probes). Images were obtained using an inverted fluorescence microscope combined with a digital camera and Meta-Morph software. For quantification, MetaVue software was used as described in detail in (Cicha et al. 2008).

To downregulate FoxO1 and FoxO3a expression, HUVEC were transfected with equal concentrations (50 nM each) of FoxO1 and FoxO3a siRNA or luciferase siRNA (Eurogentec, Seraing, Belgium) using Lipofect-amine 2000 (Invitrogen). The following siRNAs were used: FoxO1 sense GCG-GGC-UGG-AAG-AAU-UCA-A; FoxO3a sense GCU-CUU-GGU-GGA-UCA-UCA-A. 24 h after transfection, the cells were exposed to flow.

Results

CCN2 expression in HUVEC cultured under static con-ditions is very high, in contrast to the low expression detectable in the endothelial lining of healthy vessels. Therefore, CCN2 expression was analyzed in cells pre-exposed to physiologic levels of laminar flow (10 dyne/cm^2). Compared to cells cultured under static conditions, CCN2 is downregulated under these conditions (Fig. 1 and (Cicha et al. 2008)). Following 18 h pre-conditioning with laminar flow, the cells were treated with a specific inhibitor of PI3K, LY294002 (10 μM), for additional 2 h at flow. Inhibition of PI3K–AKT signaling led to a robust upregu-lation of CCN2 protein expression (Fig. 1). CCN2 expression was detectable in a perinuclear Golgi localiza-tion, as observed earlier (Samarin et al. 2009). These data thus showed that negative regulation of CCN2 by PI3K–AKT signaling was not restricted to microvascular endo-
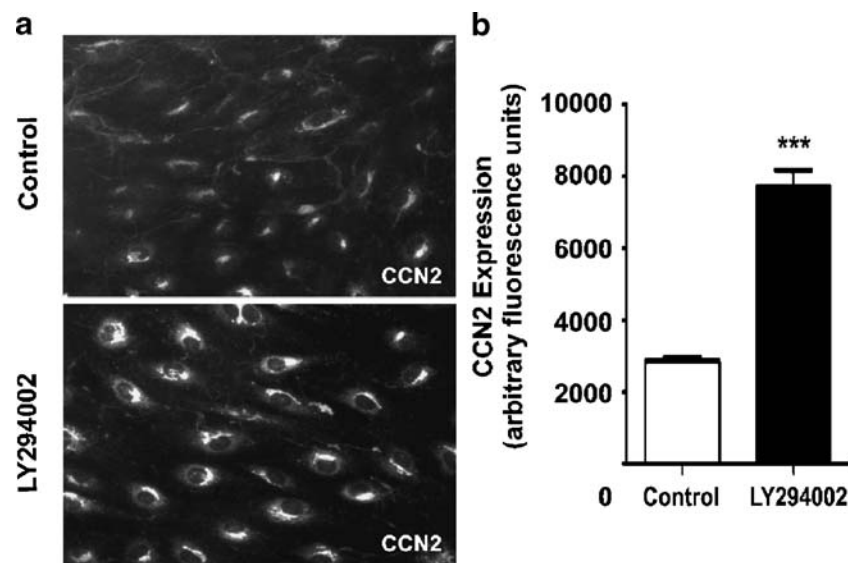
Fig. 1 Inhibition of PI3K increases CCN2 expression in HUVEC exposed to laminar shear stress. **a** HUVEC were seeded in flow-through cell culture slides and exposed to steady laminar shear stress for 20 h at 10 dyne/cm^2. For the last 2 h of flow, PI3K–AKT signaling was inhibited by treatment with LY294002 (10 μM). Protein expression was determined by immunofluorescence. *Photos* are representa-tive of two independent experiments performed in duplicate. **b** Data quantification: six to seven images at objective magnification 20× were taken for every experiment, and analyzed using MetaVue software. The thresholded protein expression levels were expressed as arbitrary fluorescence units. ***$P<0.001$; *t*-test vs. untreated controls, $n=2$ with duplicate cell culture slides

thelial cells but also observed in primary cultures of HUVEC studied under physiological flow conditions.

Transcription factors of the FoxO family are activated upon inhibition of PI3K–AKT signaling. Our previous data obtained in microvascular endothelial cells showed that these transcription factors are involved in the regulation of CCN2 expression (Samarin et al. 2009; Komorowsky et al. 2009). Therefore, we used an siRNA approach to analyze the role of FoxO transcription factors in HUVEC. FoxO1 was readily detectable in HUVEC by Western blot analysis and by immunocytochemistry (Fig. 2 and data not shown), whereas FoxO3a was barely detectable. However, it has been reported that these FoxO proteins can compensate one another (Potente et al. 2005). To avoid this potential compensating effect, we used a mixture of siRNAs directed against FoxO1 and FoxO3a.

Treatment of HUVEC exposed to laminar flow with the PI3K inhibitor LY294002 for 2 h led to nuclear localization of FoxO1 in all cells transfected with luciferase siRNA (Fig. 2, upper panel). In samples transfected with FoxO1/3a siRNA, nuclear localization of FoxO1 was only detectable in few cells (Fig. 2, lower panel). These cells expressed CCN2 at levels comparable to control HUVEC. By contrast, downregulation of FoxO1 by siRNA transfection was associated with a reduced expression of CCN2.

We have reported earlier that CCN2 is downregulated under laminar flow and upregulated when HUVEC are exposed to non-uniform shear stress (Cicha et al. 2008).

Interestingly, FoxO1 was barely detectable in cells exposed to laminar shear stress, whereas it was localized to the nucleus in cells exposed to non-uniform shear stress (Fig. 3). Nuclear localization of FoxO1 correlated with an increased expression of CCN2 under *in vivo*-like hemody-namic conditions, suggesting a role of FoxO proteins in CCN2 regulation by non-uniform shear stress.

Discussion

Regulation of CCN2 expression via PI3K–AKT signaling shows a remarkable cell type- and context-dependence. As outlined in the introduction, there is good evidence in the literature that activation of AKT is related to increased CCN2 expression. However, the molecular details of this activation remain to be defined. AKT activity is modulated by several binding partners which modulate signaling downstream of PI3K (Franke 2008). It is also unknown which of the multiple downstream mediators of AKT can actually lead to the increased CCN2 gene expression.

Our previously published data summarized in this report, provide evidence that PI3K–AKT negatively regulates CCN2 expression in endothelial cells. Upregulation rather than downregulation of CCN2 upon inhibition of PI3K–AKT signaling was observed in mouse microvascular endothelial cell lines obtained from heart or kidney (Samarin et al. 2009). This indicated that active AKT
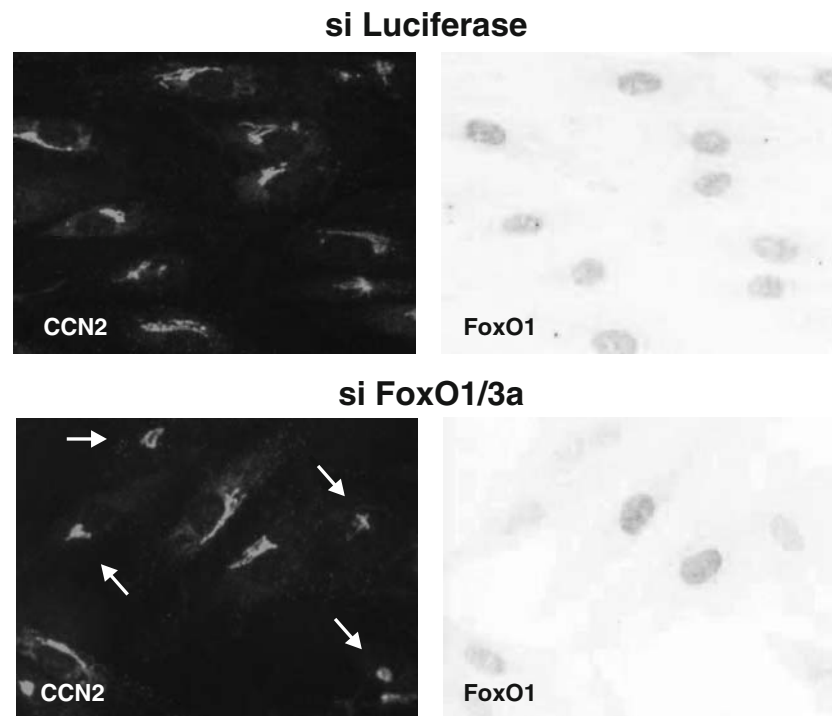
si Luciferase

si FoxO1/3a

Fig. 2 FoxO1 is involved in CCN2 regulation in HUVEC. HUVEC were transfected with siRNA against luciferase or FoxO1 and FoxO3a 24 h after seeding. The next day the cells were exposed to laminar shear stress for 18 h and then treated with LY294002 (10 μM for 2 h). CCN2 and FoxO1 were detected by indirect immunofluorescence. Images showing nuclear FoxO1 expression were *inverted*. *Arrows* indicate cells with barely detectable FoxO1 expression and low levels of CCN2 expression. *Color figures* showing the merged images are provided as "Supplemental data"

Laminar shear stress
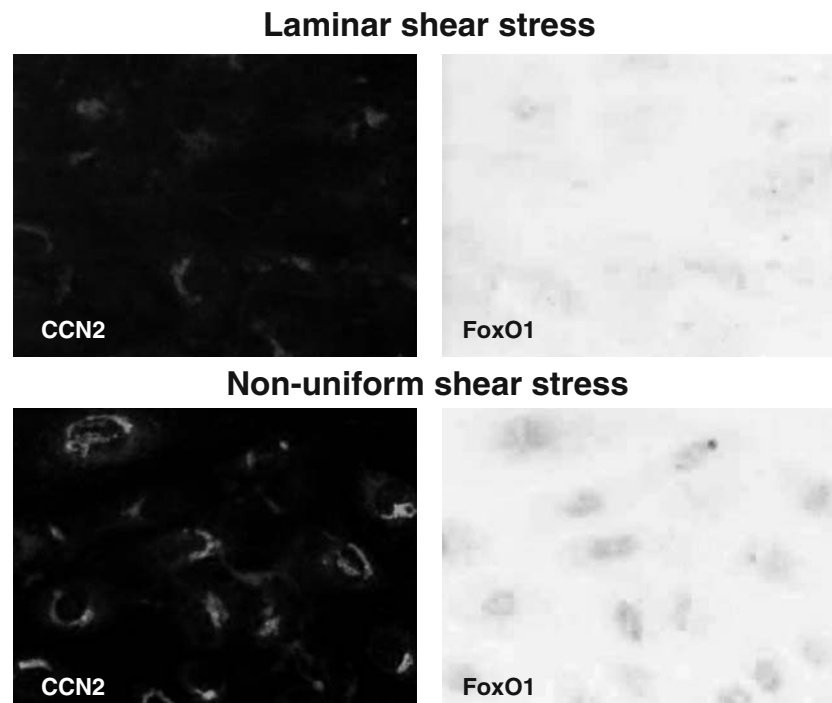

Non-uniform shear stress

Fig. 3 FoxO1 expression depends on flow conditions and is associated with CCN2 induction by non-uniform shear stress. HUVEC were seeded in bifurcating flow-through slides as described in detail in (Cicha et al. 2008). Cells were exposed to flow for 20 h. *Photos* were taken in areas of laminar shear stress (*upper panel*) or areas of non-uniform shear stress (*lower panel*). CCN2 and FoxO1 were detected by indirect immunofluorescence. Images showing nuclear FoxO1 expression were *inverted*. *Color figures* showing the merged images are provided as "Supplemental data"

signaling prevented rather than simulated CCN2 gene expression in these cells. We now confirmed these observations in primary cultures of HUVEC exposed to laminar flow. Regarding the mechanisms of regulation, activated AKT can negatively modulate gene expression by recruiting FoxO transcription factors from the nucleus to the cytoplasm. FoxO transcription factors are important regulators of endothelial cell differentiation, angiogenesis, and maintenance of vascular homeostasis (Potente et al. 2005; Dejana et al. 2007). Interestingly, the function of FoxO proteins is highly context-specific and can differ even in endothelial cells obtained from different vascular beds (Paik et al. 2007), which may explain the apparently discrepant results obtained by Suzuma et al. (Suzuma et al. 2000). FoxO proteins localize to the cytosol upon phosphorylation, whereas dephosphorylated FoxO proteins localize to the nucleus and are active as transcription factors. Using an siRNA approach, we previously showed that these transcription factors play a central role in CCN2 induction in endothelial cells (Samarin et al. 2009; Komorowsky et al. 2009). Upregulation of CCN2 by inhibition of PI3K–AKT signaling was almost completely inhibited in the presence of siRNA against FoxO1 and FoxO3a in microvascular endothelial cells (Samarin et al. 2009) and, as shown in this study, in HUVEC. Furthermore, knock-down of FoxOs affected the induction of CCN2 by various stimuli, e.g. TGFbeta (Komorowsky et al. 2009) and hypoxia (unpublished observation).

FoxOs are not only phosphorylated by AKT but also by other kinases. AMP-activated protein kinase (AMPK) was shown to phosphorylate and thus inactivate FoxO1 in HUVEC exposed to laminar shear stress (Dixit et al. 2008). In line with these results FoxO1 was barely detectable in HUVEC exposed to laminar shear stress. However, nuclear localization was observed in HUVEC exposed to non-uniform shear stress. Whether this was related to an inhibition of AMPK remains to be investigated. Interestingly, there was a close correlation between nuclear FoxO1 localization and CCN2 expression, suggesting that the role of FoxOs in CCN2 regulation goes beyond PI3K–AKT signaling.

FoxO proteins as transcription factors have been shown to bind to coactivator and corepressor complexes. These interactions are modulated by the posttranscriptional modification state of FoxOs, which involves acetylation and ubiquitination besides phosphorylation (Vogt et al. 2005). There are several putative FoxO binding sites in the core promoter of CCN2 (Gomis et al. 2006), but the complexes formed with other transcripiton factors have not yet been analyzed in detail. The molecular details of FoxO-mediated upregulation of CCN2 thus remain to be investigated.

In accordance with our results, microarray data showed that upon overexpression of constitutively active AKT, CCN2 was downregulated, whereas active FoxO1 upregulated CCN2 in HUVEC (Daly et al. 2004). In human keratinocytes, CCN2 was identified as a member of a FoxO-Smad synergism group, i.e. proteins which were upregulated by TGFbeta in a Smad- and FoxO-dependent manner (Gomis et al. 2006). These data indicate that FoxO proteins may play an important role in the regulation of CCN2 in other cell types besides endothelial cells.

In summary, regulation of CCN2 expression via PI3K–AKT signaling varies depending on the cell type and the cell culture conditions. In endothelial cells, activation of PI3K–AKT suppresses CCN2 expression, whereas inhibition of AKT activates FoxO transcription factors, which serve as potent inducers of CCN2 gene expression. FoxO proteins are thus key regulators which determine the effects of the activation of PI3K–AKT signaling on CCN2 gene expression.

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