NOX4 mediates activation of FoxO3a and matrix metalloproteinase-2 expression by urotensin-II

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ABSTRACT **The vasoactive peptide urotensin-II (U-II) has been associated with vascular remodeling in different cardiovascular disorders. Although U-II can induce reactive oxygen species (ROS) by the NADPH oxidase NOX4 and stimulate smooth muscle cell (SMC) proliferation, the precise mechanisms linking U-II to vascular remodeling processes remain unclear. Forkhead Box O (FoxO) transcription factors have been associated with redox signaling and control of proliferation and apoptosis. We thus hypothesized that FoxOs are involved in the SMC response toward U-II and NOX4. We found that U-II and NOX4 stimulated FoxO activity and identified matrix metalloproteinase-2 (MMP2) as target gene of FoxO3a. FoxO3a activation by U-II was preceded by NOX4-dependent phosphorylation of c-Jun NH(2)-terminal kinase and 14-3-3 and decreased interaction of FoxO3a with its inhibitor 14-3-3, allowing MMP2 transcription. Functional studies in FoxO3a-depleted SMCs and in FoxO3a$^{-/-}$ mice showed that FoxO3a was important for basal and U-II–stimulated proliferation and vascular outgrowth, whereas treatment with an MMP2 inhibitor blocked these responses. Our study identified U-II and NOX4 as new activators of FoxO3a, and MMP2 as a novel target gene of FoxO3a, and showed that activation of FoxO3a by this pathway promotes vascular growth. FoxO3a may thus contribute to progression of cardiovascular diseases associated with vascular remodeling.**

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INTRODUCTION

Remodeling of the pulmonary or systemic vasculature is a hallmark of many progressive cardiovascular diseases and is characterized by a delicate balance among proliferation, apoptosis, and extracellular matrix modifications. Urotensin-II (U-II) is a small, cyclical, vasoactive peptide that has been found elevated in several cardiovascular diseases associated with vascular remodeling, including systemic and pulmonary hypertension, congestive heart failure, and atherosclerosis (Djordjevic and Gorlach, 2007; Ross *et al.*, 2010). Although considered to be the most potent endogenous vasoconstrictor discovered to date (Maguire and Davenport, 2002), the exact pathophysiological relevance of U-II in these disorders is not completely understood. Recent evidence indicates that U-II can activate cells of the vascular wall to proliferate and migrate, and to generate reactive oxygen species (ROS) by induction of a NOX4-dependent NADPH oxidase (Sauzeau *et al.*, 2001; Djordjevic *et al.*, 2005; Papadopoulos *et al.*, 2008). In addition, U-II has been implicated to act on extracellular matrix (ECM) composition, because it induces the expression of plasminogen activator inhibitor-1 in pulmonary artery smooth muscle cells (SMC; Djordjevic *et al.*, 2005) and increases collagen synthesis in endothelial cells (Wang *et al.*, 2004). The exact mechanisms linking U-II to ECM decomposition and remodeling of the vascular wall, however, are so far not resolved.

ECM decomposition is mediated primarily by matrix metalloproteinases (MMPs), a family of more than 20 enzymes that are responsible for the cleavage of different ECM components. MMPs are usually present in latent forms. Activation of MMPs occurs by proteolytic cleavage and by complex protein–protein interactions. In addition

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Abbreviations used: BrdU, 5-bromo-2′-deoxyuridine; DBE, DAF16 binding elements; FHRE, Forkhead response element; FoxO, Forkhead Box O; JNK, c-Jun NH(2)-terminal kinase; MAP, mitogen-activated protein; MMP, matrix metalloproteinase; MST1, mammalian Ste20-like kinase-1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI3, phosphoinositide 3; ROS, reactive oxygen species; shRNA, short hairpin RNA; SMC, smooth muscle cell(s); U-II, urotensin-II; WT, wild-type.

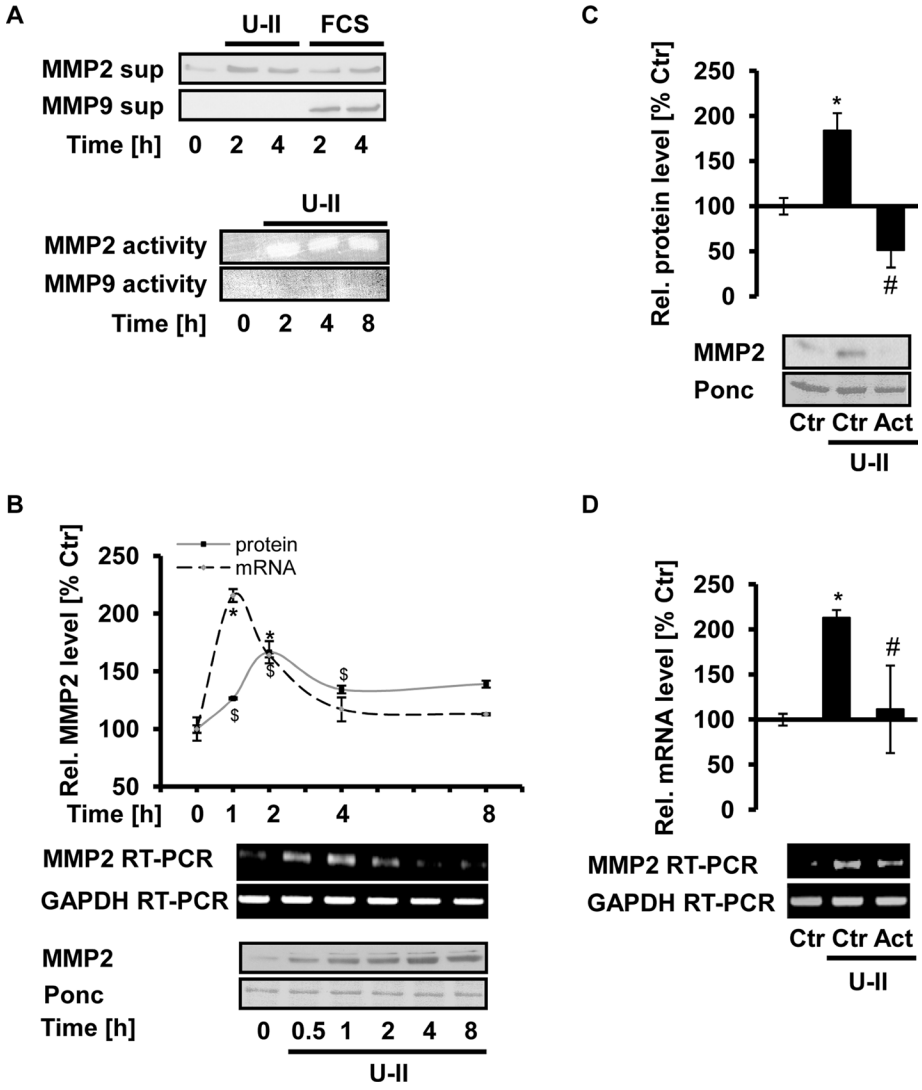

FIGURE 1: U-II stimulates MMP2 expression and activity. (A) Pulmonary artery SMC were stimulated with U-II (100 nM) or 10% fetal calf serum (FCS) for different time periods. Supernatants were subjected to Western blot analysis using antibodies against MMP2 or MMP9 or to zymography to determine MMP2 or MMP9 activity. (B) SMC were stimulated with U-II for the indicated time periods. MMP2 mRNA and protein were determined by RT-PCR using primers for MMP2 or GAPDH or by Western blot analyses using an antibody against MMP2, respectively. PonceauS staining served as loading control. Data are presented as relative change to unstimulated cells (100%; n = 3, *p < 0.05 vs. unstimulated cells [0]). (C and D) SMC were pretreated with actinomycin D (Act, 5 μM) or dimethyl sulfoxide (DMSO) (Ctr) for 1 h and stimulated with U-II for 2 h. (C) Western blot analyses were performed with an antibody against MMP2. PonceauS staining served as loading control. (D) RT-PCR was performed using primers for MMP2 or GAPDH. Data are presented as relative change to control (100%) (n = 3, *p < 0.05 vs. unstimulated Ctr, #p < 0.05 vs. U-II–stimulated Ctr).

to matrix degradation and remodeling, MMPs have been implicated in cell growth, migration, angiogenesis, and arteriogenesis, but also in apoptosis, thus making them important factors governing structural alterations of the vascular wall (McCawley and Matrisian, 2001).

The gelatinases MMP2 and MMP9 have been frequently associated with vascular remodeling processes in atherosclerosis (Back *et al.*, 2010), and MMP2 has been suggested to play an important role in remodeling of the ECM and the vascular wall of lung vessels in pulmonary hypertension (Hassoun, 2005; Lepetit *et al.*, 2005; Raffetto and Khalil, 2008). Increased MMP2 expression due to polymorphisms in the MMP2 promoter seems to be relevant for the risk of cardiovascular events (Volcik *et al.*, 2010). The regulatory pathways underlying the expression of MMP2 in the vasculature, however, are not completely resolved.

The family of Forkhead Box O (FoxO) transcription factors, in particular, FoxO1, FoxO3a, and FoxO4, has been implicated to play a critical role in the control of proliferative and apoptotic processes. Initial work has linked metabolic insulin signaling and life-span extension with these transcription factors in multiple species (Accili and Arden, 2004; Calnan and Brunet, 2008). Under these conditions, phosphorylation of FoxO proteins by Akt can result in the association with 14-3-3 proteins and sequestration of this complex inactively in the cytosol (Calnan and Brunet, 2008). Increasing evidence suggests that FoxO transcription factors are important survival factors under severe stress conditions and regulate expression of several genes involved in stress resistance, cell survival, and apoptosis also in the cardiovascular system (Maiese *et al.*, 2009). Although there is strong awareness that FoxO transcription factors are essentially involved in redox signaling (Storz, 2011), there are only limited data linking ROS-generating NADPH oxidases to FoxO transcriptional activity in the vasculature. Because MMP2 expression and activity have been associated with ROS signaling, we hypothesized that U-II as an activator of NOX4 may regulate FoxO signaling and MMP2 expression.

Here we provide evidence that FoxO activity was stimulated by U-II and a NOX4-dependent pathway. We identified MMP2 as a novel target gene of FoxO3a, and showed that FoxO3a was instrumental in the proliferative response toward U-II not only in vitro, but also ex vivo as demonstrated in vessels derived from FoxO3a$^{-/-}$ mice. Thus our data provide evidence for a novel pathway linking NOX4 to FoxO3a, MMP2 expression, and vascular proliferation and suggest an important role of FoxO3a in vascular remodeling.

RESULTS

U-II increases MMP2 activity and expression

First, we aimed to set up an in vitro model of vascular remodeling by stimulating human pulmonary artery SMC with 100 nM U-II, a dose we previously have shown to be sufficient to activate these cells (Djordjevic *et al.*, 2005).

In this setting we determined the activity of the gelatinases MMP2 and MMP9 as important mediators of vascular remodeling. U-II rapidly stimulated the secretion and activity of MMP2, whereas MMP9 secretion and activity appeared unaffected under these conditions (Figure 1A). In contrast, exposure to 10% serum increased

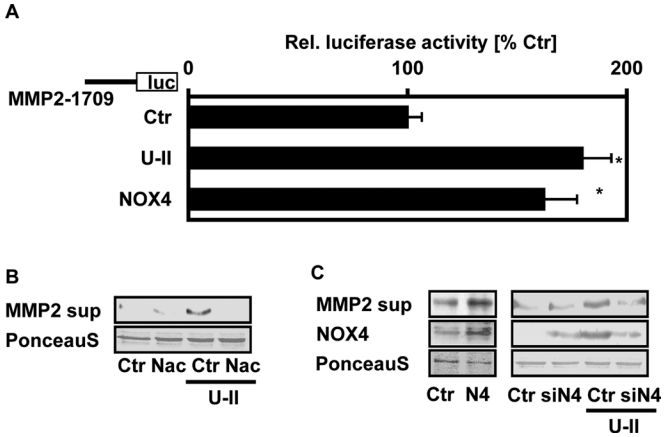

FIGURE 2: NOX4 and ROS promote U-II–induced MMP2 expression. (A) Pulmonary artery SMC were transfected with a human MMP2 promoter luciferase construct (MMP2-1709) and cotransfected with a NOX4 expression vector or control vector (Ctr). Cells were stimulated with U-II (100 nM) for 8 h or left untreated. Luciferase activity under control conditions was set to 100% (n = 5; *p < 0.05 vs. Ctr). (B) SMC were preincubated with NAC (10 mM) for 30 min or left untreated and stimulated with U-II for 4 h. Western blot analysis was performed with cell culture supernatants (sup) using an MMP2 antibody. PonceauS staining served as loading control. (C) SMC were transfected with vectors coding for NOX4 or for shRNA against NOX4 (siN4) or the respective control vectors (Ctr), and stimulated with U-II for 4 h. Western blot analyses were performed with antibodies against MMP2 and NOX4. PonceauS staining served as loading control. Blots are representative of three independent experiments.

the release of both MMP2 and MMP9, suggesting that U-II acts specifically on MMP2 regulation.

Subsequently, we focused on MMP2 and found that U-II rapidly increased MMP2 mRNA and protein levels, peaking at 1 and 2 h, respectively (Figure 1B). Up-regulation of MMP2 mRNA and protein by U-II was prevented by actinomycin D (Figure 1, C and D), indicating a transcriptional response. In line with this result, reporter gene analyses with a luciferase construct driven by the human MMP2 promoter showed that U-II robustly increased MMP2 promoter activity (Figure 2A).

NOX4 mediates MMP2 up-regulation by U-II

We previously showed that U-II is able to increase ROS production via a NOX4-dependent NADPH oxidase (Djordjevic *et al.*, 2005). Therefore we determined whether MMP2 expression by U-II was driven by a redox-sensitive pathway and NOX4. Treatment with the antioxidant *N*-acetylcysteine (NAC) diminished MMP2 induction by U-II (Figure 2B).

In support, depletion of NOX4 with short hairpin RNA (shRNA) decreased MMP2 protein levels in the presence of U-II, whereas overexpression of NOX4 increased MMP2 protein levels as well as MMP2 promoter activity (Figure 2, A and C), indicating that NOX4 is involved in the regulation of MMP2 expression under these conditions.

U-II increases the activity of FoxO transcription factors

In the next step we aimed to dissect the molecular mechanisms regulating MMP2 expression by NOX4 and U-II. Bioinformatic analysis of the MMP2 promoter identified a previously unrecognized putative binding site for FoxO transcription factors at −278 to −294 base pairs upstream of the translational start site. This site was conserved in human, mouse, and rat. Using two different reporter gene constructs to determine FoxO activity, either driven by consensus sites of the *Caenorhabditis elegans* FoxO homologue DAF-16 or by Forkhead responsive elements from the FasL gene, we found that exposure to U-II as well as NOX4 overexpression increased FoxO activity (Figure 3A). In contrast, other known activators of SMC, such as PDGF (Figure 3A) or transforming growth factor-β1 (unpublished data), did not stimulate FoxO activity although they were able to increase activity of other transcription factors such as hypoxia-inducible factors by activating corresponding reporter genes (data not shown).

FoxO3a mediates up-regulation of MMP2 by U-II

To test whether FoxO transcription factors are involved in the regulation of MMP2 in the presence of U-II, we used shRNAs to decrease the levels of FoxO1, FoxO3a, and FoxO4. Depletion of FoxO1 and FoxO4 did not substantially affect FoxO activity or MMP2 levels in the presence of U-II (Figure 3). In contrast, knockdown of FoxO3a, which specifically down-regulated FoxO3a but had no effect on the expression of FoxO1 and FoxO4 (data not shown), almost completely abrogated FoxO activity and subsequently induction of MMP2 by U-II (Figure 3), suggesting that FoxO3a is prominently involved in the regulation of MMP2 under these conditions.

To further substantiate these observations, we tested the involvement of FoxO3a in the regulation of MMP2 expression. FoxO3a overexpression increased MMP2 mRNA levels and promoter activity similar to U-II (Figure 4, A and B). Interestingly, U-II was able to further induce MMP2 promoter activity in FoxO3a-overexpressing cells, indicating a partially additive effect. In contrast, expression of an inactive FoxO3a mutant lacking the transactivation domain prevented MMP2 promoter activation by U-II, but did not affect basal promoter activity (Figure 4B). Importantly, mutation of the putative FoxO binding site abolished MMP2 promoter activation by FoxO3a, NOX4, and U-II (Figure 4B). In line with these results, chromatin immunoprecipitation analyses confirmed increased binding of FoxO3a to the MMP2 gene in response to U-II (Figure 4C), indicating that MMP2 is a novel NOX4-dependent target gene of FoxO3a.

FoxO3a binding to 14-3-3 is diminished by U-II

Next we aimed to further analyze the upstream mechanisms leading to MMP2 transcription by FoxO3a and investigated the association of FoxO3a with 14-3-3. This chaperone is known to regulate FoxO transcriptional activity because it can interact with FoxO transcription factors in the cytosol and prevent them from DNA binding in the nucleus. However, 14-3-3 can be phosphorylated by c-Jun NH(2)-terminal kinase (JNK), which results in the release of FoxO3a (Calnan and Brunet, 2008). Immunoprecipitation analysis revealed that FoxO3a interacts with 14-3-3 under basal conditions in SMC (Figure 5A). In the presence of U-II, however, this interaction was substantially reduced. Interestingly, U-II was able to rapidly stimulate the phosphorylation of 14-3-3 (Figure 5B), whereas treatment with the JNK inhibitor SP612005 diminished 14-3-3 phosphorylation (Figure 5C). This response was dependent on NOX4 because depletion of NOX4 prevented not only phosphorylation of JNK, but also of 14-3-3 by U-II (Figure 5D). Importantly, application of SP612005 restored the interaction of FoxO3a with 14-3-3 in the presence of U-II (Figure 5A) and subsequently diminished the activation of FoxO by U-II (Figure 5E). In support, U-II specifically increased FoxO3a levels in the nucleus, whereas depletion of NOX4 or treatment with SP612005 decreased nuclear FoxO3a content (Supplemental Figure 1). Consequently, SP612005 treatment prevented MMP2 promoter activation and protein induction by U-II (Figure 5, E and F).

On the contrary, treatment with the phosphoinositide 3 (PI3)-kinase inhibitor LY294002, which inhibited phosphorylation of Akt

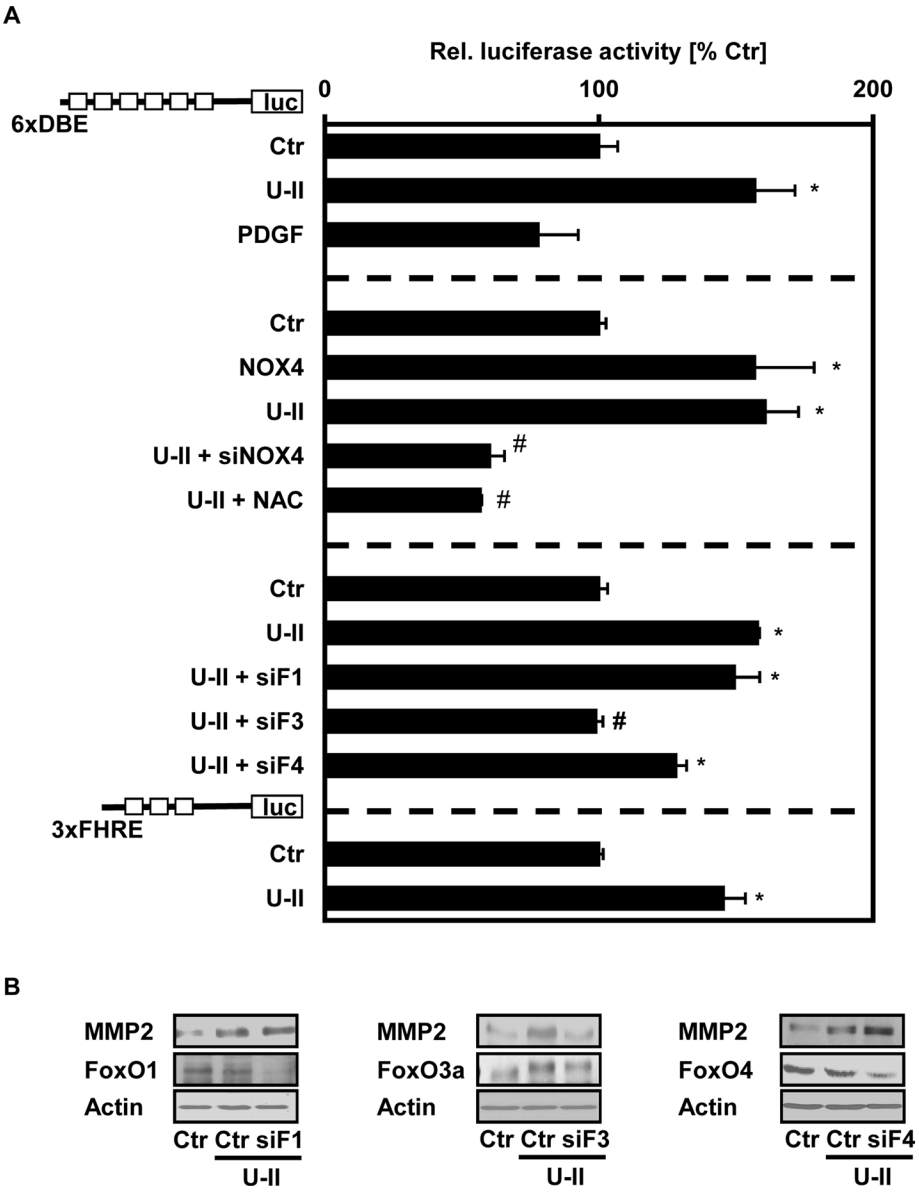

FIGURE 3: FoxO is activated by U-II and mediates MMP2 expression. (A) Pulmonary artery SMC were transfected with luciferase constructs containing either six DBE (6xDBE) or three Forkhead responsive elements from the FasL gene (3xFHRE) in front of the SV40 promoter. Cells were stimulated with U-II (100 nM) or PDGF (10 nM) for 8 h or left untreated. In some cases SMC were cotransfected with plasmids coding for NOX4 or for shRNA against NOX4 (siNOX4), FoxO1 (siF1), FoxO3a (siF3), or FoxO4 (siF4) or with respective control vectors, or were pretreated with NAC (10 mM) for 30 min. Luciferase activity under control conditions (Ctr) was set equal to 100% (n = 4; *$p < 0.05$ vs. Ctr or $^{\#}p < 0.05$ vs. U-II-stimulated Ctr). (B) SMC were transfected with vectors coding for shRNA against FoxO1 (siF1), FoxO3a (siF3), or FoxO4 (siF4) or for control shRNA (Ctr) and exposed to U-II for 4 h before Western blot analysis using antibodies against MMP2, FoxO1, FoxO3a, or FoxO4. Actin levels served as loading control. Blots are representative of three independent experiments.

(Supplemental Figure 2A) but not of JNK (Figure 5C), did not substantially affect MMP2 protein levels induced by U-II (Figure 5F and Supplemental Figure 2A), nor did it affect MMP2 promoter activity (data not shown). In addition, SP612005 treatment did not affect Akt phosphorylation (Supplemental Figure 2A). In addition, neither of the mitogen-activated protein (MAP) kinase inhibitors SB202190 and PD98059 could decrease MMP2 levels in response to U-II (Figure 5F), further confirming the importance of JNK in the regulation of MMP2.

Collectively, these findings suggest that U-II stimulates NOX4-dependent activation of JNK and subsequent phosphorylation of 14-3-3, thereby diminishing the interaction of FoxO3a with this chaperone and allowing its transcriptional activation.

FoxO3a promotes vascular proliferation in response to U-II and NOX4

In a subsequent step we aimed to investigate functional consequences of FoxO3a activation by U-II. Because U-II and NOX4 have been shown to stimulate SMC proliferation (Djordjevic *et al.*, 2005) we examined the role of FoxO3a in this response. To this end, FoxO3a was depleted from SMC by shRNA, and the proliferative response was measured by incorporation of 5-bromo-2′-deoxyuridine (BrdU). Whereas U-II or NOX4 overexpression stimulated, as expected, BrdU incorporation, depletion of FoxO3a reduced basal proliferation and diminished U-II– as well as NOX4-induced SMC proliferation (Figure 6, A–C). In addition, expression of the transcriptionally inactive FoxO3a mutant inhibited BrdU incorporation, decreased the number of SMC (Figure 6, C and D), and diminished SMC viability (Supplemental Figure 2B) in the presence of U-II. On the contrary, overexpression of FoxO3a increased SMC proliferation comparable to the effects of U-II or NOX4 (Figure 6D).

Importantly, although SMC isolated from wild-type (WT) aortae showed increased proliferation in the presence of U-II, this response could not be observed in SMC isolated from FoxO3a$^{-/-}$ mice (Figure 7A).

Increased proliferative activity mediated by FoxO3a was not related to modulation of apoptotic activity because overexpression of neither WT nor inactive FoxO3a affected activity of caspases 3 or 7 (Supplemental Figure 2C).

Interestingly, application of an MMP2 inhibitor decreased U-II– as well as FoxO3a-dependent proliferative activity (Figure 6, E and F), suggesting that MMP2 as a novel target gene of U-II and FoxO3a may act as one effector regulating the proliferative response toward U-II, NOX4, and FoxO3a.

The importance of FoxO3a for MMP2 expression and vascular proliferation was further underlined by studies in aortae and pulmonary arteries isolated from WT and FoxO3a$^{-/-}$ mice. In both vessel types, U-II stimulated MMP2 mRNA levels (Figure 7B), indicating the validity of our in vitro findings in the ex vivo situation. MMP2 expression was completely diminished in vessels derived from FoxO3a$^{-/-}$ mice, confirming that MMP2 is regulated by FoxO3a.

We then tested sprouting capacity of vascular rings derived from both vessel types. U-II significantly augmented sprouting from WT aortae or pulmonary arteries (Figure 7C). Basal as well

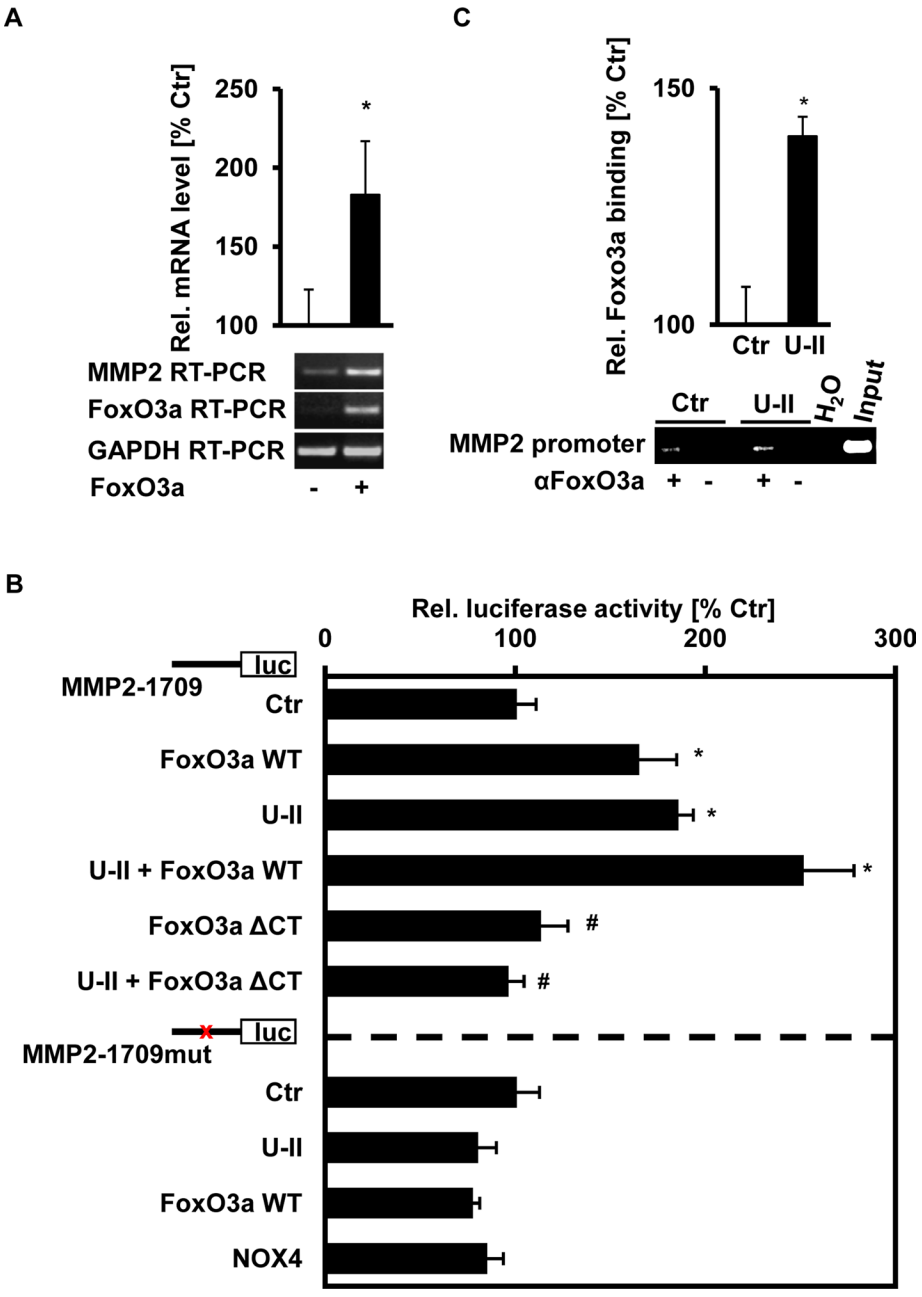

FIGURE 4: FoxO3a regulates MMP2 transcription and promoter activity. (A) Pulmonary artery SMC were transfected with a vector coding for FoxO3a or with control vector (–). RT-PCR was performed using primers for MMP2, FoxO3a, or GAPDH. Data are presented as relative change to control vector (100%; n = 3, *p < 0.05 vs. control vector [–]). (B) SMC were cotransfected with luciferase constructs containing either the WT MMP2 promoter (MMP2-1709) or the MMP2 promoter mutated at the FoxO consensus sequence (MMP2-1709mut) and with plasmids coding for WT (FoxO3a WT) or inactive (FoxO3a ΔCT) FoxO3a or for NOX4. Cells were stimulated with U-II (100 nM) for 8 h or left untreated. Luciferase activity under control conditions (Ctr) was set equal to 100% (n = 3; *p < 0.05 vs. Ctr; #p < 0.05 vs. U-II–stimulated Ctr). (C) SMC were stimulated with U-II for 2 h and subjected to chromatin immunoprecipitation with an antibody against FoxO3a. PCR amplifying a 310–base pair fragment of the proximal MMP2 promoter region containing a putative FoxO binding site was performed. Data are presented as relative change to control (Ctr, 100%; n = 3, *p < 0.05 vs. Ctr).

as U-II–stimulated sprouting was greatly diminished, however, in FoxO3a$^{-/-}$ vessels compared with WT controls (Figure 7C). These ex vivo findings support our in vitro findings that FoxO3a is required for basal as well as U-II–induced vascular proliferation.

DISCUSSION

In this study we identified FoxO3a as a critical element promoting vascular proliferative responses and MMP2 expression in response to U-II. We also deciphered the molecular pathways underlying this finding and demonstrated that activation of FoxO3a by U-II is mediated by NOX4, ROS, and subsequent phosphorylation of JNK and 14-3-3.

MMP2 is a novel FoxO3a target gene

Our data provide evidence that U-II can increase MMP2 expression in SMC by a transcriptional mechanism involving the NADPH oxidase NOX4. Whereas only limited data are available to date about the role of U-II in the regulation of MMPs, our findings complement earlier studies linking the NADPH oxidase component p47phox to MMP2 in response to cyclical stretch in mouse SMC (Grote *et al.*, 2003). Although p47phox does not seem to be required for NOX4-dependent oxidase activity, our findings that NOX4 is important for regulation of MMP2 by U-II is supported by a study demonstrating that NOX4 contributes to MMP2 induction by insulin-like growth factor-1 in SMC (Meng *et al.*, 2008), further confirming the relevance of NADPH oxidases for MMP2 regulation.

Our study further details the link between NOX4 and MMP2 by demonstrating that MMP2 transcription by U-II, NOX4, and ROS is mediated by the transcription factor FoxO3a, which bound to a Forkhead response element (FHRE) in the MMP2 5′ flanking region, indicating that MMP2 is a target gene of FoxO3a. The importance of FoxO3a as a regulator of U-II–induced MMP2 expression was further confirmed by our findings that depletion of neither FoxO1 nor FoxO4 significantly affected FoxO activity or MMP2 expression in the presence of U-II. In support, U-II increased FoxO3a binding to the MMP2 gene, and MMP2 was decreased or even absent in vessels derived from FoxO3a$^{-/-}$ mice even in the presence of U-II.

Although FoxO transcription factors have been considered to bind to identical DNA binding sites, recent evidence suggests that these factors have partially overlapping but also nonredundant functions as is best demonstrated by the different phenotypes of FoxO1$^{-/-}$, FoxO3a$^{-/-}$, and FoxO4$^{-/-}$ mice (Monsalve and Olmos, 2011).

Although the exact mechanisms governing DNA binding specificity of FoxO proteins are still under investigation and beyond the scope of this article, there is increasing evidence that several mechanisms may contribute to target gene specificity of FoxO proteins. For example, it has been suggested that each FoxO member has a different optimal DNA sequence specificity at the 5′-end of DAF16 binding

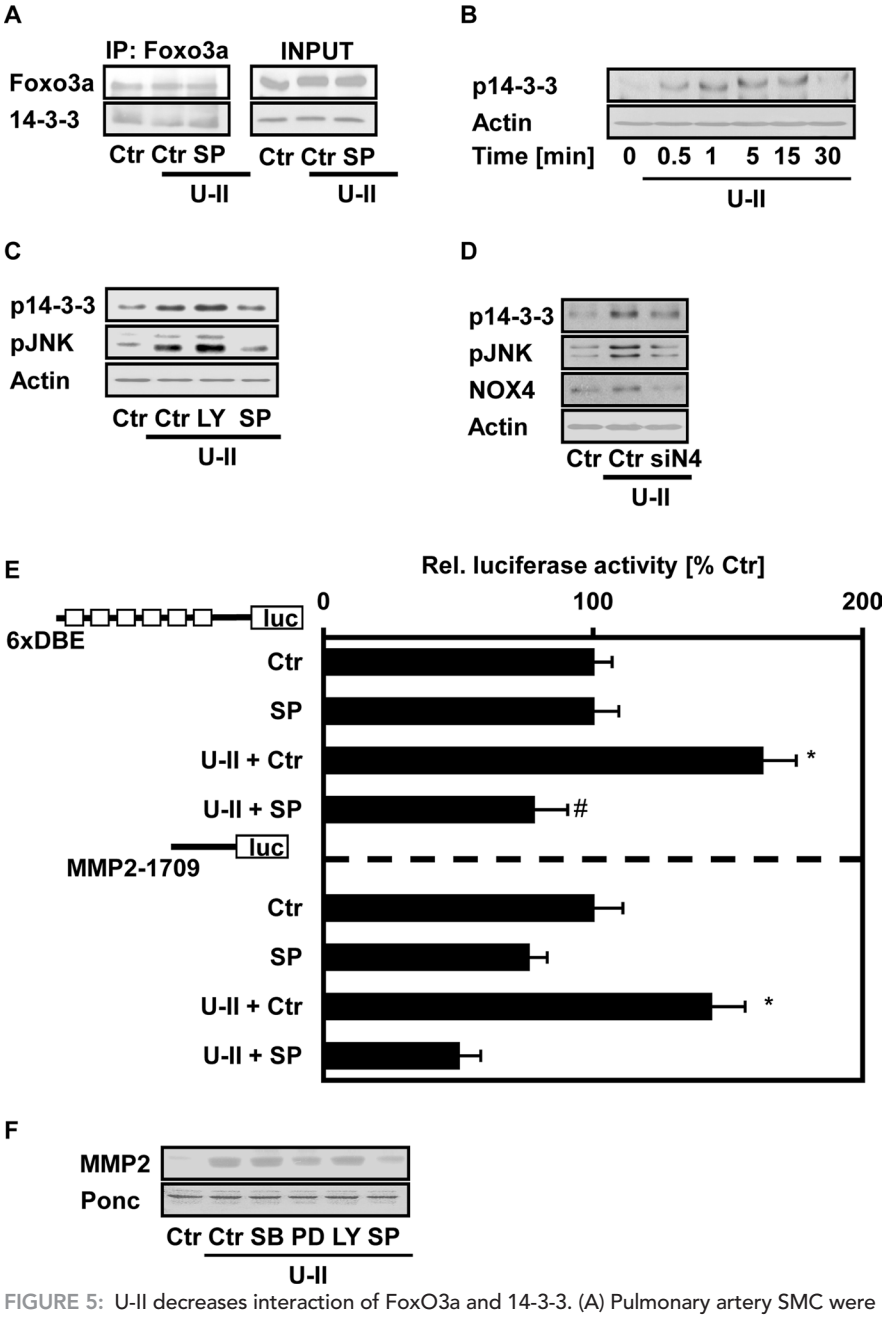

FIGURE 5: U-II decreases interaction of FoxO3a and 14-3-3. (A) Pulmonary artery SMC were stimulated with U-II (100 nM) for 15 min in the presence or absence of the JNK inhibitor SP600125 (SP, 25 μM). Immunoprecipitation was performed with an antibody against FoxO3a and revealed with an antibody against 14-3-3. Blots are representative of three independent experiments. (B) SMC were stimulated with U-II for increasing time periods. Western blot analysis was performed with antibodies against phosphorylated 14-3-3 (p14-3-3) and actin. Blots are representative of three independent experiments. (C) SMC were preincubated with SP600125 (SP, 25 μM), the PI3-kinase inhibitor LY294002 (LY, 10 μM), or DMSO (Ctr) for 30 min or (D) were transfected with shNOX4 (siN4) or control vector (Ctr) and stimulated with U-II for 5 min. Western blot analyses were performed using antibodies against phosphorylated 14-3-3 (p14-3-3), JNK (pJNK), NOX4, and actin. Blots are representative of three independent experiments. (E) SMC were transfected with luciferase constructs containing six DBE in front of the SV40 promoter (6xDBE) or the MMP2 promoter (MMP2-1709), preincubated with SP or DMSO (Ctr) for 30 min, and stimulated with U-II for 8 h. Luciferase activity under control conditions (Ctr) was set equal to 100% (n = 3; *$p < 0.05$ vs. Ctr or $^{\#}p < 0.05$ vs. U-II–stimulated Ctr). (F) SMC were preincubated for 30 min with the MEK1 inhibitor PD98059 (PD, 20 μM), the p38 MAP kinase inhibitor SB202190 (SB, 20 μM), LY294002 (LY, 10 μM), or SP600125 (25 μM) or with solvent (Ctr) and stimulated with U-II for 4 h. Western blot analysis was performed using antibodies against MMP2 and actin. Blots are representative of three independent experiments.

elements (DBE) that may relate to the differential target gene recognition for each FoxO member (Xuan and Zhang, 2005). Thus one may speculate that the MMP2 gene contains in addition to the FoxO consensus site additional sequences that favor binding of FoxO3 over other FoxO proteins, thus explaining that FoxO3a selectively regulated MMP2 expression in response to U-II. In addition, there are accumulating results indicating that FoxO3a activity can be regulated by a multitude of protein–protein interactions and posttranslational modifications including phosphorylation, acetylation, and ubiquitination, which in turn affect its localization, protein stability, and in particular specific DNA binding and transcriptional activity (Obsil and Obsilova, 2010). Thus it is tempting to hypothesize that U-II is able to stimulate a specific regulatory program in SMC that favors FoxO3a activation.

The identification of MMP2 as a target gene of FoxO3a extends previous studies reporting an involvement of FoxO3a in the regulation of MMP3 in endothelial cells (Lee *et al.*, 2008) and of MMP9 in cancer cells (Storz *et al.*, 2009). Whereas we identified MMP2 as a direct target of FoxO3a, however, MMP9 and MMP3 expression were indirectly regulated by FoxO3a. Similarly, FoxO4 up-regulated MMP9 expression in SMC stimulated by TNF-α by an indirect mechanism (Li *et al.*, 2007), whereas it did not affect MMP2 expression, further supporting our observations that FoxO3a, and not FoxO4, is primarily involved in the regulation of MMP2 by U-II.

NOX4 and U-II promote FoxO transcriptional activity

Our study further showed that NOX4 and ROS, which acted downstream of U-II to induce MMP2 transcription, were importantly involved in FoxO activation by U-II. Although ROS have been previously related to activation of FoxO transcription factors (Essers *et al.*, 2004; Liu *et al.*, 2005), the sources of ROS generation leading to activation of FoxOs are not well elucidated.

Here we provide evidence that a NOX4-dependent NADPH oxidase known to play an important role in delivering ROS as signaling molecules in the vasculature was instrumental in the activation of FoxO3a by promoting the phosphorylation of JNK and subsequently of 14-3-3 in response to U-II independently of the PI3-kinase/Akt pathway. Binding of FoxO3a to 14-3-3 has been shown to sequester this transcription factor inactively in the cytoplasm (Calnan and Brunet, 2008), whereas phosphorylation of 14-3-3 by JNK disrupted binding of FoxO3a to 14-3-3 (Sunayama *et al.*, 2005). These findings support our observations that U-II reduced the interaction between FoxO3a and 14-3-3 and that inhibition of JNK restored this interaction.

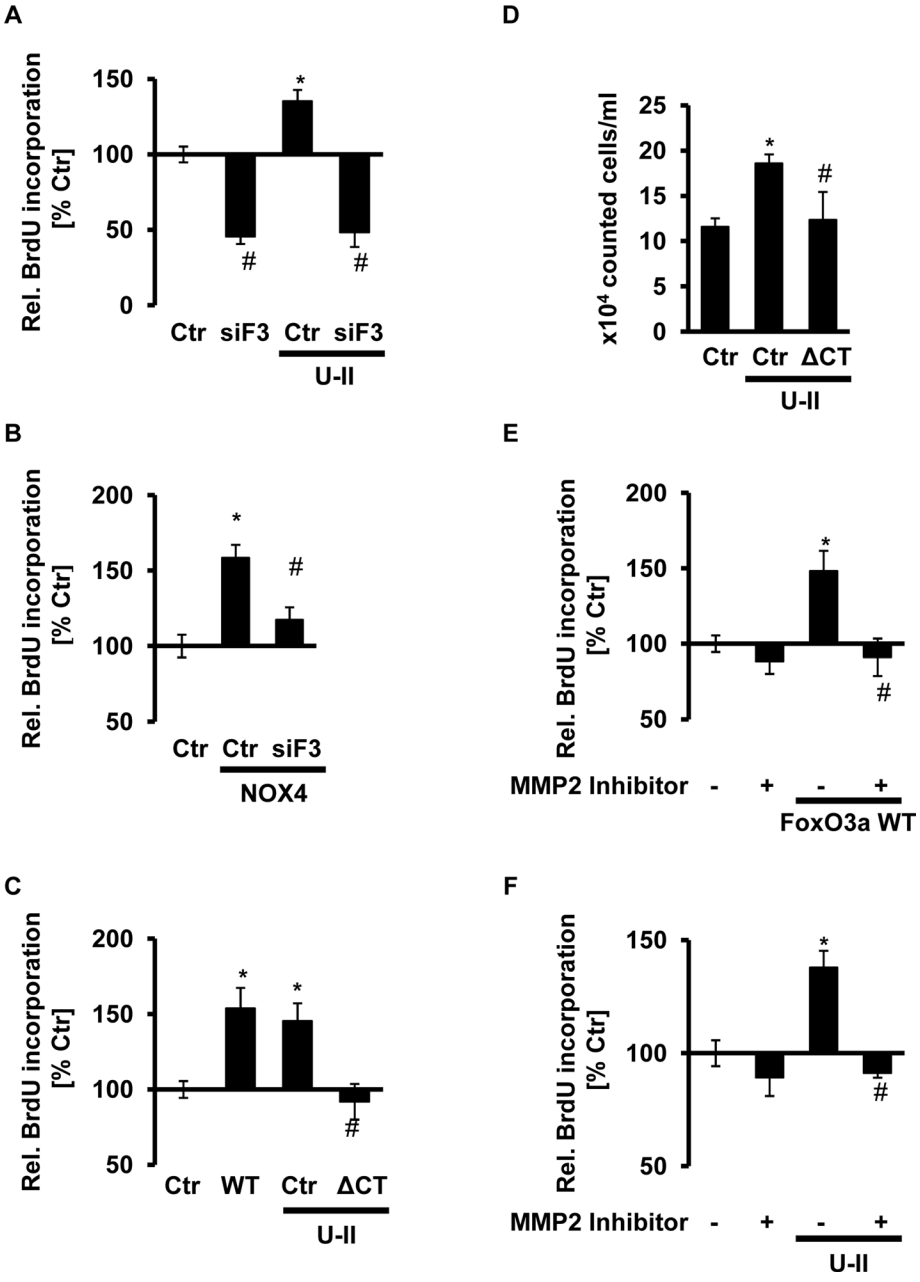

FIGURE 6: FoxO3a promotes U-II–induced SMC proliferation. (A and B) Pulmonary artery SMC were transfected with vectors coding for shRNA against FoxO3a (siF3) or control shRNA (Ctr), and stimulated with U-II (100 nM) for 2 h (A), or were cotransfected with a vector for NOX4 (B). DNA synthesis was evaluated by BrdU incorporation. Data are shown as relative change to control (Ctr, 100%; n = 3, *$p < 0.05$ vs. Ctr, #$p < 0.05$ vs. U-II–stimulated Ctr or NOX4 overexpression). (C and D) SMC were transfected with vectors for WT or inactive (ΔCT) FoxO3a and stimulated with U-II for 2 h. DNA synthesis was evaluated by BrdU incorporation, and control was set to 100% (C), or cell numbers were determined using a hemocytometer (D) (n = 3, *$p < 0.05$ vs. Ctr, #$p < 0.05$ vs. U-II–stimulated Ctr). (E and F) SMC were transfected with a vector encoding FoxO3a (E) or were stimulated with U-II for 2 h (F), after treatment with an MMP2 inhibitor (20 μM) (+) or DMSO (–) for 30 min. BrdU incorporation was evaluated. Data are shown as relative change to DMSO control (Ctr, 100%; n = 3, *$p < 0.05$ vs. Ctr, #$p < 0.05$ vs. overexpression of FoxO3a or U-II–stimulated Ctr).

ROS-dependent activation of JNK has been described to directly phosphorylate FoxO4 resulting in nuclear translocation and enhanced transcriptional activity (Essers *et al.*, 2004). Because the residues targeted by JNK in FoxO4 are not conserved in FoxO3a (Huang and Tindall, 2007), direct ROS- and JNK-dependent phosphorylation of FoxO3a appears unlikely to be involved in the response to U-II. ROS, however, have been shown to promote phosphorylation of FoxO3a by the mammalian Ste20-like kinase-1 (MST1), thereby blocking the interaction of FoxO3a with 14-3-3 and enhancing nuclear localization of FoxO3a (Huang and Tindall, 2007). Because MST1 is expressed in SMC (Ono *et al.*, 2005), and can be activated by JNK (Huang and Tindall, 2007), it cannot be excluded at that point that MST1 may also contribute to the NOX4-ROS-JNK–dependent activation of FoxO3a by U-II. In addition, ROS have been described to modulate FoxO activity by acetylation, thereby either increasing the levels of acetylated FoxO proteins in the nucleus and hindering their transcriptional activity, or promoting deacetylation of FoxO proteins by activation of NAD-dependent deacetylases such as sirtuins, thus enhancing FoxO-dependent gene transcription (Brunet *et al.*, 2004; Frescas *et al.*, 2005). Of note, overexpression of the deacetylase Sirt1 further increased U-II and FoxO3a induced MMP2 promoter activity (data not shown), suggesting that full activation of FoxO3a-dependent gene transcription may be limited by acetylation in our cellular system.

FoxO3a regulates vascular proliferation

The functional importance of U-II– and NOX4-induced activation of FoxO3a and the subsequent induction of MMP2 was further highlighted by our findings that both FoxO3a and MMP2 were critically involved in controlling the proliferative response of SMC toward U-II and NOX4. These findings provide further insights into our previous observation that U-II is able to enhance SMC proliferation in a ROS-dependent manner involving NOX4 (Djordjevic *et al.*, 2005). Although we previously have shown that U-II can increase SMC proliferation involving (in addition to JNK) MAP kinases and Akt (Djordjevic *et al.*, 2005), our new data indicate that only inhibition of JNK (but not of MAP kinases or the PI3-kinase/Akt pathway) can prevent induction of MMP2 by U-II, indicating that JNK-regulated MMP2 expression is sufficient to promote proliferation in response to U-II.

Extending earlier studies showing that MMP2 can promote migration and proliferation of SMC (Rauch *et al.*, 2002), our findings now provide evidence that FoxO3a as a regulator of MMP2 expression also promoted the proliferative response toward U-II not only in vitro but also in vivo. Strikingly, growth of isolated FoxO3a$^{-/-}$ SMC as well as vascular outgrowth from vessel rings derived from aortae or pulmonary arteries from FoxO3a$^{-/-}$ mice was dramatically reduced when compared with the response in WT SMC or vessel rings.

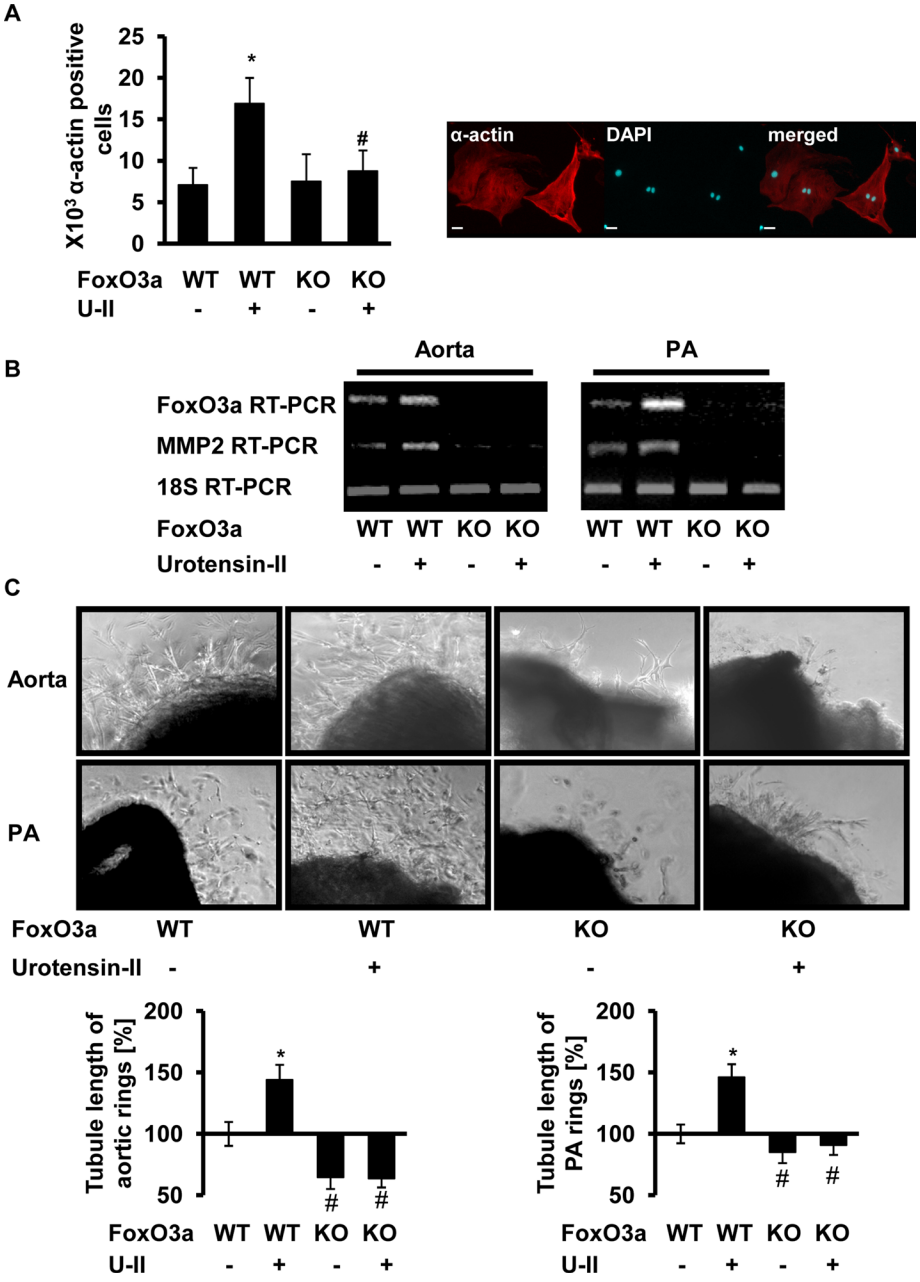

FIGURE 7: FoxO3a is required for vascular outgrowth. (A) SMC were isolated from FoxO3a WT or FoxO3a knockout (KO) mice and stimulated with U-II (100 nM) for 72 h. Cell number was counted in a hemocytometer. To confirm the presence of SMC, cells were fixed and stained with an antibody against SMC α-actin (red). Nuclei were counterstained with DAPI (blue). Size bar = 20 μm for all panels. (B) Aortae or pulmonary arteries (PA) from FoxO3a WT or FoxO3a knockout (KO) mice were stimulated with U-II for 4 h. RT-PCR was performed from mRNA isolated from these vessels using primers for MMP2, FoxO3a, and 18S. (C) Vessel rings from aortae or pulmonary arteries (PA) from FoxO3a WT or KO mice were cultured in Matrigel with or without U-II to allow vessel sprouting. Tubule length was quantified using ImageJ software. Data are presented as relative change to control (WT, 100%; n = 3, *p < 0.05 vs. WT, #p < 0.05 vs. U-II–stimulated WT).

Together with our preliminary findings that MMP2 can also be induced by U-II in endothelial cells, and that FoxO3a depletion prevents U-II–stimulated endothelial cell tube formation (data not shown), our findings indicate that FoxO3a is required for vascular proliferation under basal and U-II–stimulated conditions.

Our findings that FoxO3a mediates NOX4- and U-II–induced vascular proliferative responses are supported by studies reporting that FoxO3a depletion reduces survival of murine myoblasts toward H_2O_2 (Li *et al.*, 2008) and that FoxO4 depletion prevents SMC migration by TNF-α (Li *et al.*, 2007). FoxO3a has also recently been shown to promote tumor cell invasion in Matrigel (Storz *et al.*, 2009).

In contrast, forced expression of constitutively active, but not WT, FoxO3a has been reported to diminish SMC proliferation and increase apoptosis and even cell death (Abid *et al.*, 2005; Lee *et al.*, 2007), whereas FoxO3a deficiency protected against prolonged hindlimb ischemia (Potente *et al.*, 2005). Whereas the exact reasons underlying these apparently conflicting results still need to be elucidated, they may be at least partially related to our observation that the effect of FoxO3a on SMC proliferation is dose-dependent: Overexpression of low to moderate levels of FoxO3a as shown throughout the study increased SMC proliferation and viability, possibly by promoting cell-cycle progression (data not shown), but had no effect on caspase activity, whereas expression of high amounts of FoxO3a decreased SMC proliferation again and conversely enhanced the levels of apoptosis markers (data not shown). A similar dose-dependent effect of FoxO3a was observed with regard to MMP2 promoter activation, suggesting that FoxO3a dose-dependently regulates target gene expression, which may relate to the apparently conflicting data of the role of FoxO3a in controlling cell survival, proliferation, or cell death.

In support of our study it was recently shown that depletion of FoxO3a diminished early onset of microglia proliferation in response to oxygen-glucose deprivation, a condition associated with oxidative stress (Shang *et al.*, 2009). Subsequently, upon prolonged stress, FoxO3a depletion reduced caspase activation and promoted microglia survival. In contrast, expression of inactive FoxO3a or depletion of FoxO3a under our experimental conditions reduced U-II–induced proliferation and survival but did not affect caspase activity. In support, FoxO3a$^{-/-}$ SMC did not respond with increased proliferation to stimulation with U-II. These findings indicate that, at early or mild stages of stress, FoxO3a is required to initiate a proliferative cellular program, but that, upon onset of severe or prolonged stress, FoxO3a may contribute to a proapoptotic program shift.

Interestingly, a recent report suggested that direct binding of FoxO3a to FHRE may enhance transcriptional activation of genes involved in vascular remodeling in endothelial cells, whereas the proapoptotic functions of FoxO3a appeared to be mediated independently of FHRE binding (Czymai *et al.*, 2010). Our findings that FoxO3a selectively increases MMP2 expression via binding to an

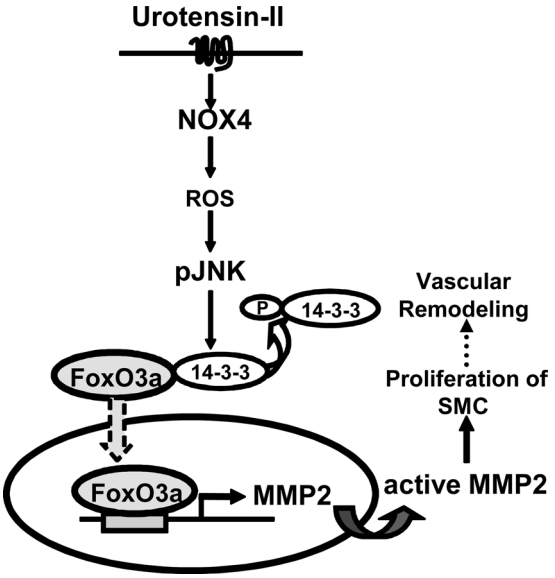

FIGURE 8: FoxO3a activation by U-II leads to vascular proliferation. The scheme summarizes the pathways described: U-II activates FoxO3a by NOX4-mediated ROS production and phosphorylation of JNK and 14-3-3. This pathway leads to up-regulation of MMP2 and enhanced proliferation of SMC and may thus play an important role in vascular remodeling.

FHRE, together with our observations that FoxO3a is relevant for MMP2 expression and vascular outgrowth ex vivo, support the idea that FoxO3a may be critically involved in vascular remodeling processes in our model system. This idea is also in line with in vivo studies reporting elevated levels of the FoxO3a activator U-II in patients prone to vascular remodeling due to coronary heart disease (Chai *et al.*, 2010), type 2 diabetes with progressive diabetic retinopathy and carotid atherosclerosis (Suguro *et al.*, 2008), and congenital heart disease (Simpson *et al.*, 2006).

In summary, our findings delineate FoxO3a as a critical signaling molecule promoting a proliferative response of vascular cells activated by U-II and NOX4 (Figure 8). Activation of FoxO3a by U-II involved NOX4-dependent phosphorylation of JNK and 14-3-3 and resulted in the up-regulation of MMP2 and enhanced SMC proliferation, whereas SMC proliferation was blunted in the absence of FoxO3a in vitro and in vivo.

As FoxO3a is a convergence point of growth factor and stress stimulus signaling, modulation of FoxO3a signaling may provide an interesting therapeutic opportunity to combat remodeling processes in the course of cardiovascular diseases.

MATERIALS AND METHODS

Reagents

cis-9-octadecenoyl-*N*-hydroxylamide (MMP2 inhibitor-I) and PDGF were obtained from Calbiochem (Darmstadt, Germany). All other chemicals were obtained from Sigma (Taufkirchen, Germany).

Cell culture

Human pulmonary artery SMC were obtained from Lonza (Wuppertal, Germany) and cultured as recommended to passage 11. A7r5 rat SMC (ATCC CRL-1444, ATCC, Wesel, Germany) were cultured in DMEM (Life Technologies, Karlsruhe, Germany) with 10% fetal calf serum. Cells were serum-starved for 16 h before experiments.

Mouse SMC were isolated from aorta as previously described (Hirakawa *et al.*, 1999; Ray *et al.*, 2001). Mice were killed, and the aorta was quickly removed. Blood vessels were carefully cleaned from connective tissue, and the endothelium was removed by gently rubbing. The remaining part of the aorta was cut into small pieces of approximately 2 mm in length. Tissues were incubated in an enzyme solution containing 1.5 mg/ml collagenase type II (Worthington Biochemical Corporation, Lakewood, NJ). Enzymatic digestion was terminated after a 4-h incubation by the addition of 5 ml of culture medium (DMEM with 4.5 g/l glucose, 10% fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin). The SMC suspension was centrifuged twice at 300 × *g* for 5 min at room temperature, and the pellet was resuspended in culture medium. The cell suspension was plated on 35-mm glass-bottom dishes and incubated at 37°C in a humidified atmosphere of 5% CO_2.

Plasmids

A 1709–base pair fragment of the human MMP2 5′ flanking region from −1943 to −235 base pairs relative to the translation start site was amplified by PCR and subcloned into pGL3-BASIC (Promega, Mannheim, Germany) to create pGL3-MMP2-1709. Mutation of a FoxO binding site (–278 to –294 base pairs) at position –285/286 using the QuikChange Mutagenesis Kit (Promega) revealed pGL3-MMP2-MUT. Vectors for WT FoxO3a (FLAG-FoxO3aWT), inactive FoxO3a with a deletion of the transactivation domain (HA-FoxO3aWTΔCT), the vector encoding for NOX4 and the shRNA against NOX4, and the luciferase constructs pGL3-6xDBE and pGL3-3xFHRE have been described (Furuyama *et al.*, 2000; Tran *et al.*, 2002; Calnan and Brunet, 2008; Diebold *et al.*, 2010). shRNA against FoxO3a was created using the siSTRIKE U6 Hairpin Cloning System (Promega). A random control shRNA was already described (Petry *et al.*, 2006). All plasmids were confirmed by sequencing.

Transfection and luciferase assays

SMC were transfected as described (Djordjevic *et al.*, 2004). Transfection efficiency was 60–70%. Because human SMC do not efficiently express luciferase constructs, rat A7r5 SMC were used for reporter gene assays and transfected with calcium phosphate as described (Djordjevic *et al.*, 2004). A Renilla luciferase expression vector was cotransfected to adjust for variations in transfection efficiencies.

RNA extraction and RT-PCR

RNA was extracted using an RNeasy Kit (Qiagen, Hilden, Germany). RT-PCR was performed with the following exon-spanning primers: human MMP2: forward 5′-CAGATGCCTGGAATGCCATC-3′, reverse: 5′-GCAGCCTAGCCAGTCGGATT-3′; human FoxO3a: forward: 5′-TCTGTCCCAGATCTACGAGTG-3′, reverse: 5′-CATCAGGGTTGATGATCCACC-3′; mouse MMP2: forward: 5′-CAGACTCCTGGAATGCCATC-3′; reverse: 5′-GCAGCCCAGCCAGTCTGATT-3′; mouse FoxO3a: forward: 5′-CCCCATCGGGGTTGATGATCCACC-3′; reverse: 5′-TTTGTCCCAGATCTACGAGTG-3′. These primers revealed only PCR products of the expected size (for MMP2, 161 base pairs) thus ruling out amplification of DNA (expected size 2631 base pairs). No amplification products were observed without addition of RT. Sequences of PCR products of the expected size were verified by sequence analyses.

Western blot analysis and zymography

Western blot analyses were performed as described (Djordjevic *et al.*, 2005) using antibodies against MMP2 (Oncogene Research Products, Boston, MA), NOX4 (Diebold *et al.*, 2010), 14-3-3β/α, FoxO3a, phosphorylated JNK, phosphorylated Akt (all obtained from Cell Signaling, Frankfurt, Germany), and phosphorylated

14-3-3β/α (Abcam, Cambridge, UK). Equal sample loading was evaluated by reprobing membranes with a β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Goat anti–mouse or anti–rabbit immunoglobulin (Calbiochem) was used as secondary antibody. The enhanced chemiluminescent Western blotting system was used for detection. For zymography, 50 µg of protein from cell lysates was loaded onto a polyacrylamide gel containing 0.1% gelatin A (Invitrogen, Karlsruhe, Germany). After electrophoresis, gels were washed in 2.5% Triton X-100 and stained with Coomassie Blue.

Immunofluorescence

Immunofluorescence was performed as described (Petry *et al.*, 2006) using an antibody against FoxO3a (Cell Signaling). Nuclei were counterstained with DAPI (Invitrogen). The secondary antibodies coupled to Alexa 488 or 594 were obtained from Mobitec (Göttingen, Germany).

Immunoprecipitation

Immunoprecipitation was performed as previously described (Petry *et al.*, 2006) using antibodies against FoxO3a and 14-3-3β/α (Cell Signaling).

Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed in A7r5 cells as described (Bonello *et al.*, 2007). Chromatin was precipitated using an antibody against FoxO3a (Cell Signaling). From the precipitated DNA, a 310–base pair region of the MMP2 promoter was amplified by PCR with primers flanking the potential FoxO binding site (forward: 5′-CCAGCTAGGGAGCAAGAAGG-3′, reverse: 5′-GCAGGTCCTAGTAATCCCTTTG-3′).

Proliferation assays

DNA synthesis was determined by BrdU incorporation enzyme-linked immunosorbent assay (ELISA; Roche, Basel, Switzerland) as described (Djordjevic *et al.*, 2005). Briefly, SMC were seeded in 96-well plates at a density of 2000 cells/well. Cells were transfected and/or stimulated with U-II (100 nM) for 8 h. Cells were incubated with BrdU (10 µM) for 16 h, and immunodetection of incorporated BrdU was performed after incubation with a peroxidase-conjugated antibody using tetramethylbenzidine as a substrate. Absorbance was measured in an ELISA reader (Tecan, Crailsheim, Germany) at 450 nm with a reference wavelength at 690 nm.

Alternatively, equal numbers of human SMC were seeded, stimulated with U-II for 48 h, trypsinized, and counted in a standard hemocytometer. Equal numbers of primary mouse SMC were seeded in µ-slide 8-well ibiTreat chambers (Ibidi, Munich, Germany). Cells were treated with U-II for 72 h, fixed, and stained for smooth muscle cell actin as mentioned earlier in text. Nuclei were counterstained with DAPI. Smooth muscle cell actin positive and total cell numbers were determined by image analysis using ImageJ software (Wright Cell Imaging Facility, Toronto, Canada). Purity of the cell culture was >90%.

Viability and caspase assays

Cell viability was tested by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. Equal numbers of cells were transfected and seeded. After stimulation, MTT was added and precipitates were lysed with dimethyl sulfoxide. The absorbance was measured at 550 nm in a microplate reader (Tecan).

Caspase 3/7 activity was measured using the Rh110 Caspase-3/7 Assay Kit (AnaSpec, San Jose, CA) in accordance with the manufacturer's manual. Briefly, equal amounts of cells were seeded in 96-well plates and transfected. Twenty-four hours after transfection, assay buffer was added containing (Asp-Glu-Val-Asp)2–rhodamine (Rh) 110, which is cleaved by caspases 3 and 7 liberating Rh110 to generate a fluorescence signal. Fluorescence intensity is proportional to caspase 3/7 activity. Fluorescence was measured at 490 nm excitation and 520 nm emission wavelength in a 96-well plate reader (Tecan). Treatment with staurosporine (Cell Signaling) for 6 h served as positive control.

Ex vivo vascular ring sprouting assay

FoxO3a$^{-/-}$ mice (Castrillon *et al.*, 2003) were obtained from the Mutant Mouse Regional Resource Center (MMRRC) at the University of California, Davis. FoxO3a$^{-/-}$ and WT siblings (6 wk old, male, 30–35 g) were killed, and aortae and pulmonary arteries were excised and dissected into 1– to 1.5-mm-long cross-sections. Rings were placed on wells coated with Matrigel (BD Bioscience, Heidelberg, Germany) and incubated with DMEM in the presence or absence of U-II for 3 d with daily medium change. Vessel sprouting was assessed by light microscopy (Olympus, Hamburg, Germany) via Openlab Modular Software for Scientific Imaging (Improvision, Heidelberg, Germany) and was quantified with ImageJ software. All animal procedures were approved by Regierung von Oberbayern.

Statistical analysis

Data are presented as mean ± SEM. Results were compared by analysis of variance for repeated measures followed by Student–Newman–Keuls *t* test. $p < 0.05$ was considered statistically significant.

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