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TNFα-induced down-regulation of Sox18 in endothelial cells is dependent on NF-κB

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ABSTRACT

The transcription factor Sox18 plays a role in angiogenesis, including lymphangiogenesis, where it is upregulated by growth factors and directs the expression of genes encoding, e.g., guidance molecules and a matrix metalloproteinase. Conversely, we found that in human umbilical vein endothelial cells (HUVEC) Sox18 is repressed by the pro-inflammatory mediator TNFα (as well as IL-1 and LPS). Since a common feature of these mediators is the activation of the NF-κB signaling pathway, we investigated whether Sox18 downregulation is dependent on this transcription factor. Transduction of HUVEC with an adenoviral vector directing the expression of the NF-κB inhibitor IκBα prevented the downregulation of Sox18. Transient transfections of Sox18 promoter reporter genes revealed that the downregulation takes place on the level of transcription, and that the p65/RelA subunit of NF-κB was operative. Furthermore, the responsible promoter region of Sox18 is located within −1.0 kb from the transcriptional start site. The repression of Sox18 and its target genes may lead to altered formation of vessels in inflamed settings.

1. Introduction

The HMG box family member Sox18 has been associated with (lymph-)angiogenesis due to mutations in mice and humans that result in the ragged and the HLT (hypotrichosis–lymphedema–telangiectasis) phenotypes, respectively [1,2]. They are characterized by cardiovascular abnormalities, e.g., edema, cyanosis, dilation, distention and rupture of peripheral blood vessels, as well as lymphatic defects. Mutations in Sox18 affect mainly the C-terminal transactivation domain, resulting in truncated proteins with dominant-negative function [2,3]. In order to gain insight how Sox18 exerts its function(s), we and others have previously identified target genes of Sox18. The most prominent one is Prox1 that, together with the nuclear hormone receptor Coup-TFII regulates lymphatic development [4,5]. Others include VCAM-1, the μ-opioid receptor, claudin-5, MMP7, as well as the guidance molecules ephrinB2, EphA7, and semaphorin 3G, the latters suggesting a role in guided vessel formation [6–9].

In the adult organism, SOX18 expression has been detected in the heart, in capillaries within granulation tissue of skin wounds, and in the vasa vasorum in atherosclerotic lesions [10–12]. Moreover, it was found to be necessary for smooth muscle cell growth in an *in vitro* injury model [11] where it has been reported to be regulated by VEGF. The finding that Sox18 is expressed in a number of tumor cell lines and the successful inhibition of tumor angiogenesis using cell-permeable dominant-negative (dn) SOX18 mutants [13] has further supported its function in angiogenesis. Valuable mechanistic insight into the regulation of Sox18 was provided more recently by Petrovic et al., demonstrating that the transcription factor Egr1 is a key regulator of Sox18 [14]. Thereby, a cluster of three Egr1 binding sites within 89 bp of the transcription start site has been shown to be operative.

Whereas the above findings collectively support a model where Sox18 is up-regulated during different forms of vessel formation including, e.g., wound healing or tumor angiogenesis, we report here its down-regulation upon inflammatory stimulation. Most interestingly NF-κB, a transcription factor usually responsible for the cytokine-inducible expression of a plethora of pro-inflammatory genes, is responsible for the suppressive effect. Thus, Sox18 appears to be regulated counter-directional during inflammation and angiogenesis.

2. Materials and methods

2.1. Cell culture

HEK 293 cells were obtained from ATCC. RelA$^{-/-}$ MEF were kindly provided by M. Pasparakis (Cologne). Both were cultured

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in DMEM (Bio-Whittaker) supplemented with 10% FCS (Sigma), 2 mM L-glutamine (Sigma), penicillin (100 units/ml), and streptomycin (100 μg/ml). HUVEC were isolated from umbilical cords as described [15] and maintained in M199 medium (Lonza) supplemented with 20% FCS (Sigma), 2 mM L-glutamine (Sigma), penicillin (100 units/ml), streptomycin (100 μg/ml), heparin (5 units/ml), and ECGS (25 μg/ml; Promocell). HMEC-1 cells were cultured as described [16].

2.2. Plasmids

Sox18 promoter deletion constructs were generated by PCR using the Expand High Fidelity PCR System (Roche) using the primers described in the Supplementary Material, and cloned into the vector pUBT-Luc [17]. The 5xNF-κB-Luc reporter was obtained from Stratagene. Expression vectors for the different NF-κB subunits have been described [18]. Expression vectors for Egr-1, p300, and the DNA binding-deficient mutant of RelA (Y23E26) were kindly provided by E. Hofer (Vienna), C. Brostjan (Vienna), and G. Natoli (Milan), respectively. Adenoviral vectors and transduction of HUVEC has been described previously [19].

2.3. Real-time PCR

RNA was isolated and purified using the High Pure RNA Isolation Kit (Roche), cDNA was synthesized using Taqman Reverse Transcription Reagents (Roche), and PCR performed using the Rotor-Gene SYBR Green PCR Kit (Qiagen) in a Rotor-Gene Thermocycler (Qiagen). Experiments were done in triplicate, and the relative amounts of mRNAs were calculated using the Pfaffl method and normalization to ß2-microglobulin, GAPDH, or β-actin in the case of MEF. Primer sequences are given in the Supplementary Material.

2.4. Transfections and reporter gene assays

HEK293 cells were grown in 24 well plates and transfected using the calcium phosphate method [20] HUVEC were grown and transfected in 6 well plated using polyethyleneimine [21]. Transfections were done in triplicates. Luciferase levels were normalized for expression of cotransfected ß-galactosidase or EGFP.

2.5. Antibodies and Western blotting

Preparation of cell extracts and Western Blotting was done as described [22]. The α-Sox18 antibody (PA1-24474, Thermo Scientific) was used at a conc. of 2 μg/ml, the secondary antibody (ECL Anti-Rabbit, NA934V, GE Healthcare) at a 1:5000 dilution.

2.6. Bioinformatics

Comparison of the human, dog, mouse, and rat Sox18 promoter regions was done using MULAN (http://mulan.dcode.org/). Binding sites for Egr-1 and NF-κB were detected by TFSearch (http://www.cbrc.jp/research/db/TFSEARCH.html).

2.7. Statistics

Statistical analysis was performed using Microsoft Office Excel 2010. Data are reported as means + standard deviation of the mean. Differences were tested for statistical significance with a two-tailed Student's *t* test. A *p-value* of $\leqslant 0.05$ was considered significant (*), $\leqslant 0.01$ highly significant (**). All experiments were done in triplicates and repeated at least twice; a representative experiment is shown.

3. Results

Stimulation of HUVEC with the proinflammatory mediator TNFα resulted in downregulation of Sox18 mRNA starting approx. 1 h after treatment, and reaching approx. 20% of the initial levels at 4 h (Fig. 1A). IL1 and LPS showed similar effects Suppl. Fig. 1A). The other members of the subgroup F family, Sox7 and -17, were not significantly regulated (Suppl. Fig. 1B). Since we had previously identified Sox18 target genes [7], we assayed some of them and found their mRNA levels following those of Sox18 (Fig. 1B). Due to low levels of Sox18 protein in HUVEC we analyzed it in the human endothelial cell line HMEC-1 [16] and found that it was downregulated as well following TNFα stimulation (Fig. 1C).

We then tested whether the repression of Sox18 occurs at the level of transcription. Bioinformatic analysis reveled that the 7.2 kb region between Sox18 and the next gene, TCEA2, shows 6 regions that are highly conserved between species (ECR, evolutionary conserved regions), suggesting functional importance (Fig. 2A). A set of Sox18 promoter deletion constructs (Fig. 2B) was generated according to the positions of these ECRs. Transient transfections revealed that the full-length (7.2 kb) as well as the 1.0 kb fragments responded to TNFα treatment, whereas the 0.2 kb fragment showed only a small, not significant effect (Fig. 2C), suggesting that the responsible element(s) reside mainly within 1 kb upstream of the Sox18 transcription start site.

Since one of the main signaling pathways that is elicited by proinflammatory stimuli is the NF-κB pathway, and several potential NF-κB sites can be detected within the 1 kb promoter by bioinformatics (Fig. 2B), we tested whether this transcription factor plays a role in Sox18 repression. HUVEC were transduced with an adenoviral vector directing the expression of the NF-κB inhibitor IκBα. Indeed, Sox18 levels were not diminished in IκBα expressing cells upon TNFα stimulation (Fig. 3A), suggesting that the effect is dependent on NF-κB. Furthermore, transient transfection of HUVEC with different NF-κB subunits and different Sox18 promoter constructs revealed that solely the p65/RelA subunit of NF-κB was capable of downregulating Sox18 (Fig. 3B). In addition, the inducible expression of Sox18 by EGR1, a transcription factor previously shown to control its expression [14] (for positions, see Fig. 2B), could be counteracted by p65 as well, both in HEK293 cells and in HUVEC (Fig. 3C and D). Last not least, in p65 deficient mouse embryonic fibroblasts, the TNFα mediated repression of Sox18 was impaired (Fig. 3E). Together, these data demonstrate that the downregulation of Sox18 by TNFα is dependent on NF-κB, and more precisely, on its p65 subunit.

To obtain additional mechanistic insight, we tested the role of histone acetylation/deacetylation. First, HUVEC were treated with the histone deacetylase inhibitor trichostatin A (TSA), however, this agent could not prevent TNFα-mediated downregulation (Fig. 4A), whereas KLF2 that was used as a positive control responded as described [23]. Second, we cotransfected the transcriptional coactivator p300 together with the 0.2 kb and the full-length Sox18 promoter, however, it did not responded to p300 (Fig. 4B), suggesting that neither histone acetylation or deacetylation is involved in the observed effect. In order to determine whether NF-κB exerts its effect via direct binding to the Sox18 promoter we cotransfected a p65 mutant defective in DNA binding [24], and found that it could prevent downregulation similar to the wild-type, suggesting that NF-κB acts independent of DNA binding.

4. Discussion

Since NF-κB is well-established as a main regulator of the inducible expression of a multitude of genes during inflammation in endothelial cells as well as in many other cell types, its role in downregulation of Sox18 represents a rather rare activity of this

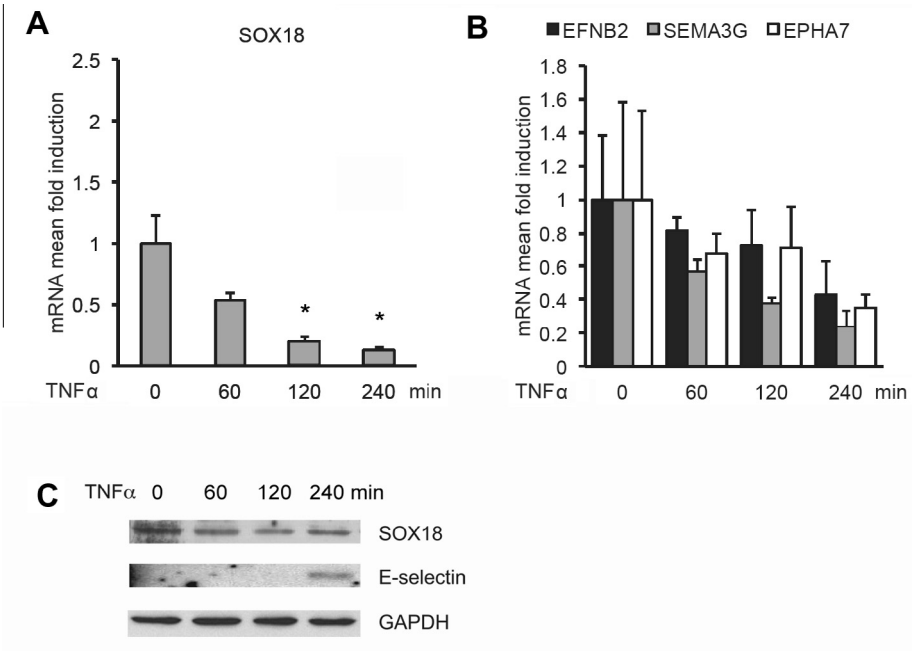

Fig. 1. Sox18 and its target genes are down-regulated by TNFα. (A) HUVEC were stimulated with TNFα for the indicated periods of time and analyzed for Sox18 mRNA by Q-PCR. (B) HUVEC were stimulated with TNFα and analyzed for expression of ephrin receptor A7, ephrin B2, and semaphorin 3G by Q-PCR. Error bars represent standard deviation of the mean; * indicates $p \leqslant 0.05$ compared to the unstimulated control. (C) Sox18 protein was analyzed in human HMEC-1 cells by Western blotting following TNFα stimulation.

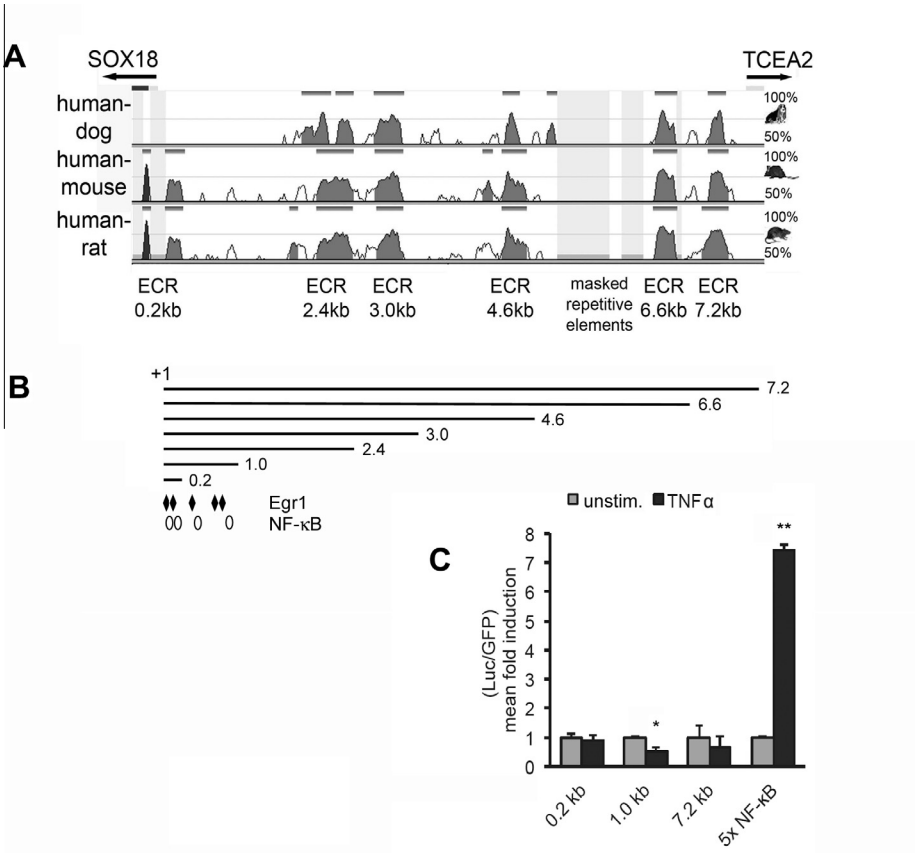

Fig. 2. Down-regulation of Sox18 by TNFα occurs on the level of transcription. (A) The 7.2 kb intergenic region upstream of Sox18 and the next gene, TCEA2, contains evolutionary conserved regions (ECRs). A comparison between human, mouse, rat, and dog is shown. (B) Sox18 promoter constructs of different length were generated according to these ECRs. Potential Egr-1 (black diamonds) and NF-κB (open circles) binding sites are shown (for the 1 kb region only of the human sequence). (C) Selected constructs were transfected into HUVEC, luciferase activity was analyzed 16 h after TNFα stimulation and normalized for the expression of cotransfected ß-gal. A NF-κB dependent reporter gene (5xNF-κB) was used as positive control for TNFα stimulation. Error bars represent standard deviation of the mean; * indicates $p \leqslant 0.05$, and ** $p \leqslant 0.01$ as compared to the unstimulated control.

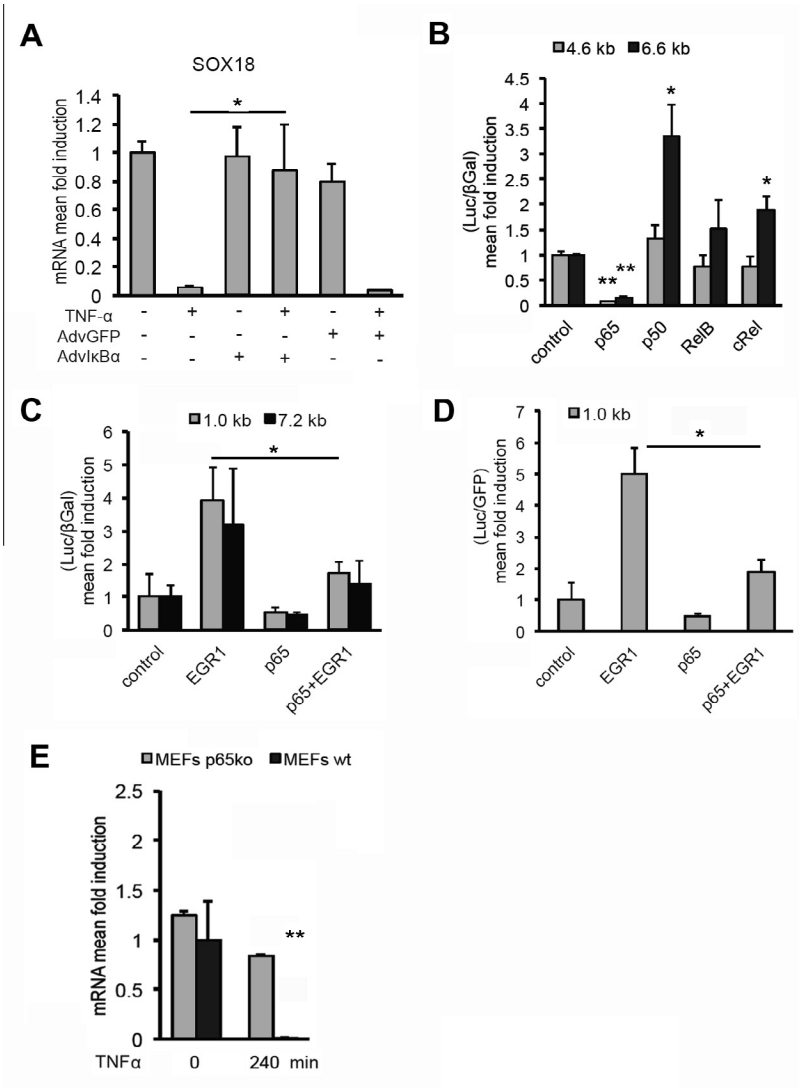

Fig. 3. The p65/RelA subunit of NF-κB is responsible for down-regulation of Sox18. (A) HUVEC were transduced with adenoviral vectors directing the expression of IκBα, or EGFP as control, stimulated with TNFα, and Sox18 analyzed by Q-PCR. * Indicates $p \leqslant 0.05$ between TNFα and TNFα + IκBα. (B) Two Sox18 promoter constructs were transiently transfected into HEK293 cells together with different NF-κB family members as indicated. Luciferase levels were determined two days later and normalized for ß-gal expression. * Indicates $p \leqslant 0.05$, and ** $p \leqslant 0.01$ as compared to the control. (C) HEK293 cells were transfected with the 1 kb and the 7.2 kb Sox18 promoter constructs together with Egr1 and p65 as indicated, and luciferase assayed as above. * Indicates $p \leqslant 0.05$ between EGR1 and EGR1 + p65. (D) HUVEC were transfected with the 1 kb Sox18 promoter construct together with Egr1 and p65, and luciferase assayed as above. * Indicates $p \leqslant 0.05$ between EGR1 and EGR1 + p65. (E) p65/RelA$^{-/-}$ MEF and control cells were stimulated with TNFα for 4 h, and analyzed by Q-PCR for Sox18 expression. Values are shown as mean fold induction relative to the wild-type unstimulated levels. **Indicates $p \leqslant 0.01$ between wt and ko cells.

transcription factor. To our knowledge, only three examples have been described in the literature in this regard, including KLF2, MIS, GADD45α and -γ [23,25,26]. In these studies, different models have been proposed for how NF-κB could down-regulate genes. These include (1) direct binding of NF-κB to the promoter and recruitment of other, inhibitory factors, or the displacement/inhibition of activating ones, (2) indirectly through sequestration by NF-κB of a coactivator that is needed by another transcription factor, (3) indirectly through DNA-independent interaction of NF-κB with a necessary transcription factor and recruitment of a repressor, or (4) NF-κB-dependent induction of an inhibitor. Suppression of KLF2 follows the third model, namely recruitment of HDAC4 by p65 to the transcription factor MEF2C [23]. Also, inhibition of MIS by TNFα in Sertoli cells follows this mechanism, namely recruitment of HDAC4 and -5 to the transcription factor SF-1 through NF-κB [26]. Downregulation of GADD45α and -γ in different cancer cells has been examined in less detail, but presumably occurs through induction of c-myc by NF-κB [25].

In the case of Sox18, the mechanism of downregulation appears to be different from the previously described ones, since inhibition of HDACs could not prevent the inhibitory effect of TNFα. Also, cotransfection of the co-activator p300 could not reverse the downregulation. In contrast to stimulation with TNFα, cotransfection of p65 had a strong effect on the 0.2 kb promoter; this might be due to overexpression of the transcription factor, which is more efficient as compared to activation through TNFα. Egr-1, a transcription factor that plays a major role in the response to several growth factors including VEGF, the main regulator of angiogenesis, has been demonstrated to be operative in the regulation of Sox18 [14]. Indeed, we observed that NF-κB can counteract the Egr-1 induced induction of Sox18 (Fig. 3C and D). The finding that the 1 kb Sox18 promoter responded stronger than the 0.2 kb fragment

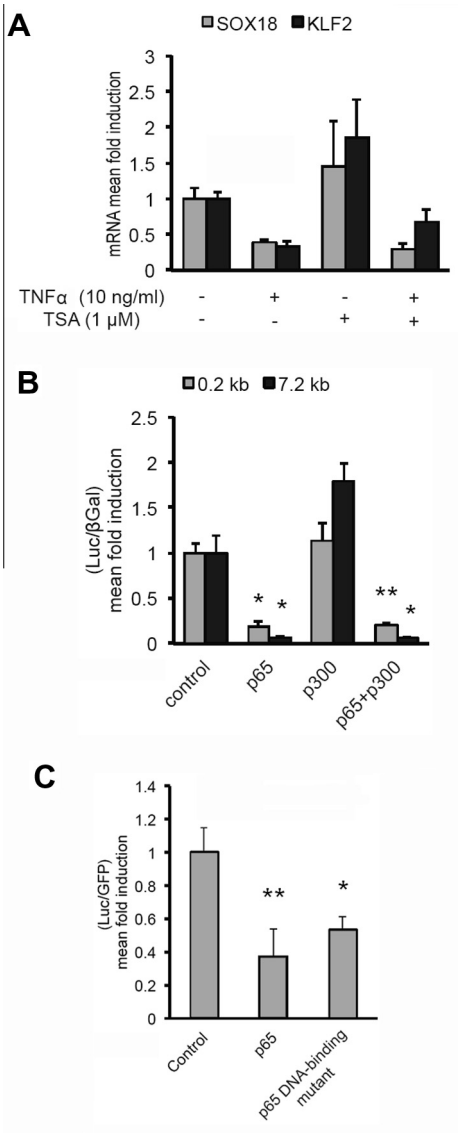

Fig. 4. Investigation of the mechanism of Sox18 downregulation. (A) HUVEC were stimulated with TNFα in the presence or absence of the histone deacetylase inhibitor trichostatin A (TSA) and analyzed for Sox18 expression using Q-PCR. No significance was detected between TNFα and TNFα + TSA stimulated samples. (B) HEK293 cells were transfected with the 0.2 and the 7.2 Sox18 promoter constructs and combinations of p65/RelA and p300 as indicated, and luciferase expression analyzed as described. * Indicates $p \leqslant 0.05$, and ** $p \leqslant 0.01$ as compared to the control. No significance was detected between p65 and p65 + p300. (C) HUVEC were transfected with the 1 kb Sox18 reporter construct together with a p65/RelA mutant defective of DNA binding (23Y/26E) and luciferase expression analyzed as described. * Indicates $p \leqslant 0.05$, and ** $p \leqslant 0.01$ as compared to the control.

could be explained by the fact that, although Petrovic et al. demonstrated the functionality of the proximal Egr-1 binding site, their 892 bp promoter responded much stronger, suggesting the presence of additional important regulatory elements in the longer promoter construct. Therefore, we favor the hypothesis that NF-κB may inhibit the activity of Egr-1, however, not through recruitment of HDACs. In contrast, the mechanism could involve physical interaction between the two transcription factors, which has been described to occur through their respective zinc finger and Rel homology domains and result in the impairment of DNA binding [27]. Although in this work the authors demonstrate inhibition of NF-κB by Egr1, it is reasonable to assume that the reverse situation may occur as well.

At this stage, we can only speculate about the biological implications. Since Sox18 is a regulator of lymphatic but also blood vessel development [4], and controls the expression of at least three different guidance molecules [7], it would imply that in an inflammatory setting these processes would be impaired. Indeed, this is supported by the observation that vessel morphology during arteriogenesis is altered upon inhibition of NF-κB, and that in type 2 diabetes that is regarded as an inflammatory disease, enhanced lymphatic microvessel density has been observed [28,29]. It will be the focus of future studies to address these questions in more detail.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.030.

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