VERSITA

Cent. Eur. J. Biol. • 5(4) • 2010 • 427-434
DOI: 10.2478/s11535-010-0031-3

Central European Journal of **Biology**

VEGF and TNF up-regulate, NSAID down-regulate SOX18 protein level in HUVEC

Research Article

Isidora Petrovic[1], Gordana Nikcevic[1], Jelena Zaric[2], Curzio Ruegg[2], Milena Stevanovic[1,*]

[1]*Laboratory for Human Molecular Genetics, Institute of Molecular Genetics and Genetic Engineering, 11010 Belgrade, Serbia*

[2]*Division of Experimental Oncology, CePO - UNIL – CHUV, CH-1066 Epalinges, Switzerland*

Received 10 November 2009; Accepted 8 March 2010

Abstract: Angiogenesis, the process of generating new blood vessels, is essential to embryonic development, organ formation, tissue regeneration and remodeling, reproduction and wound healing. Also, it plays an important role in many pathological conditions, including chronic inflammation and cancer. Angiogenesis is regulated by a complex interplay of growth factors, inflammatory mediators, adhesion molecules, morphogens and guidance molecules. Transcription factor SOX18 is transiently expressed in nascent endothelial cells during embryonic development and postnatal angiogenesis, but little is known about signaling pathways controlling its expression. The aim of this study was to investigate whether pro-angiogenic molecules and pharmacological inhibitors of angiogenesis modulate SOX18 expression in endothelial cells. Therefore, we treated human umbilical vein endothelial cells (HUVEC) with angiogenic factors, extracellular matrix proteins, inflammatory cytokines and nonsteroidal anti-inflammatory drugs (NSAID) and monitored SOX18 expression. We have observed that the angiogenic factor VEGF and the inflammatory cytokine TNF increase, while the NSAID ibuprofen and NS398 decrease the SOX18 protein level. These results for the first time demonstrate that SOX18 expression is modulated by factors and drugs known to positively or negatively regulate angiogenesis. This opens the possibility of pharmacological manipulation of *SOX18* gene expression in endothelial cells to stimulate or inhibit angiogenesis.

Keywords: *Angiogenesis • Transcription factors • SOX18 • VEGF • TNF • NSAID • Human endothelial cells*

Abbreviations

ECM - extracellular matrix;
EGR1 - early growth response 1;
FGF - fibroblast growth factor;
HUVEC - human umbilical vein endothelial cells;
NSAID - non steroidal anti-inflammatory drugs;
RT-PCR - reverse transcription polymerase chain reaction;
SOX - SRY related HMG box;
TGF - transforming growth factor;
TNF - tumor necrosis factor;
VEGF - vascular endothelial growth factor.

1. Introduction

Angiogenesis is an essential physiological process in which new blood vessels are generated from pre-existing ones. It is essential to embryonic development, organ formation, tissue regeneration and remodeling, reproduction and wound healing [1-3]. Angiogenesis is regulated by a complex interplay of growth factors,

* *E-mail: stevanov@eunet.rs*

adhesion molecules, morphogens and endogenous inhibitors, and the imbalance between these factors contributes to abnormal angiogenesis in numerous diseases [4]. Angiogenesis is also a critical event in the transition of tumors from a premalignant to a malignant state and from a dormant lesion to growing tumors [5,6]. Thus, controlling angiogenesis, either positively or negatively, is considered to be a promising strategy for the treatment of various diseases.

SOX proteins constitute a large family of diverse and well-conserved transcription factors implicated in the control of different developmental processes [7,8]. The *Sox18/SOX18* is the member of *SOX* gene family that is transiently expressed in nascent endothelial cells during embryonic development and adult neovascularization [9]. The functional importance of SOX18 protein in vascular development is demonstrated by vascular defects observed in mice and humans bearing *Sox18/SOX18* mutations. For example, mutations in *Sox18* underlie the phenotype of *ragged* mutant mouse characterized by abnormalities in its coat and cardiovascular system [10]. Furthermore, murine *Sox18* is shown to be involved in the induction of angiogenesis during wound healing and tissue repair [11]. Also, it has been demonstrated that interfering with *Sox18* function inhibits blood vessel formation and subsequent tumor growth [12]. In humans, *SOX18* mutations are associated with hypotrichosis-lymphedema-telangiectasia syndrome [13].

Numerous pro-angiogenic molecules have been identified including members of the vascular endothelial growth factor (VEGF) family, angiopoietins, transforming growth factors (TGF), platelet-derived growth factor (PDGF), tumor necrosis factor (TNF), interleukins and the members of the fibroblast growth factor (FGF) family [14]. Importantly, some of these molecules, especially growth factors and their receptors, have emerged as valuable therapeutic targets for anti-angiogenic strategies [15,16]. Although diverse transcription factors control the angiogenic phenotype of endothelial cells and represent key regulators of normal and pathological angiogenesis, only limited number of transcription factors are recognized as mediators of pro-angiogenic signals to target genes [17,18]. While SOX18 has been identified as one important transcription factor controlling blood vessel formation, little is known about extracellular cues and intracellular signaling pathways controlling its expression in endothelial cells.

Our past work has been focused on the characterization of the 5' regulatory region of the human *SOX18* gene and the identification of transcription factors involved in regulation of its expression [19,20]. We have characterized the promoter region of *SOX18* and demonstrated that specificity protein 3 (Sp3), zinc finger binding protein 89 (ZBP-89) and nuclear factor Y (NF-Y) are involved in the regulation of *SOX18* gene expression in the HeLa cell line [19,20]. Furthermore, in a search for transcription factors involved in regulation of *SOX18* gene expression that are also relevant for the control of angiogenesis, we have recently identified early growth response 1 (EGR1) as an activator of *SOX18* promoter activity [21].

The aim of this study was to investigate the effect of selected pro-angiogenic molecules and inhibitors of angiogenesis on SOX18 expression in human umbilical vein endothelial cells (HUVEC). The novel findings presented here would contribute to the unraveling involvement of SOX18 in signaling events relevant to angiogenesis.

2. Experimental Procedures

2.1 Cell culture

HUVEC were isolated from umbilical cords, as described earlier [22], using protocols approved by the Ethic Committee of the Faculty of Biology and Medicine of the University of Lausanne. HUVEC were cultured in M199 basal medium (Invitrogen, Carlsbad, CA), supplemented with 12 µg/ml bovine brain extract (Clonetics, Walkersville, MA), 10 ng/ml human recombinant epidermal growth factor (Genzyme, Cambridge, MA), 25 units/ml heparin, and 1 µg/ml hydrocortisone (Sigma Aldrich, St Louis, MO). Sub-confluent cultures were treated for different periods of time with various growth factors, TNF and NSAID with final concentrations presented at Table 1. For *de novo* cell attachment experiments, HUVEC were collected by treatment with trypsin (Invitrogen), resuspended in serum-free M199 medium, incubated for 2 hours at 37°C in suspension, and subsequently plated in 10 cm dish pre-coated with different ECM proteins (Table 1). After 3 hours, which is approximately the minimal period for full attachment of cells, cells were collected and total protein extracts were prepared, as described below.

2.2 RT-PCR

Total RNAs from HeLa and EA.hy926 cells were isolated using TRI-Reagent (Ambion, Austin, TX), while total RNA from HUVEC was isolated using RNAzol reagent (Tel-Test, Inc, Friendswood, TX) and 1 µg of each RNA was reverse transcribed using MuLV reverse transcriptase (Applied Biosystems, Foster City, CA) and random hexamers (Applied Biosystems). cDNAs were subjected to RT-PCR using Kapa Fast HS Ready Mix PCR Kit (Kapa Biosystems, Woburn, MA). *SOX18* gene was amplified using the following

primers: forward, 5′ TTCCATGTCACAGCCCCCTAG 3′, and reverse 5′ GACACGTGGGAACTCCAG 3′. GAPDH was amplified using the following primers: forward, 5′ GGACCTGACCTGCCGTCTAG 3′ and reverse, 5′ CCACCACCCTGTTGCTGTAG 3′. Products were separated on 2% agarose gel.

2.3 Western blot

Untreated and treated HUVEC were washed twice in cold PBS, and collected in RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) followed by centrifugation at 14,000 × g for 15 minutes to pellet the cell debris. Total protein extracts were separated by SDS-PAGE electrophoresis and analyzed by Western blotting using primary rabbit anti-SOX18 antibody (Santa Cruz Biotechnology, Santa Cruz, CA; sc-20100), mouse anti-actin monoclonal antibody (Sigma; A3853) and mouse anti-α-tubulin antibody (Calbiochem, Darmstadt, Germany, CP06). Immunoreactive bands were detected using HRP-conjugated anti-rabbit IgG or anti-mouse IgG and ECL detection kit (all obtained from Amersham Pharmacia Biotech, Sunnyvale, CA). Immunoblots were digitalized and quantified with ImageQuant Version 5.2 software (Amersham Pharmacia Biotech) and normalized for actin or α-tubulin values. The quantities of SOX18 protein were calculated as a percentage of control HUVEC, which was set as 100%. Data of at least two independent experiments are presented at histograms as the means ± SD. Mean values of relative SOX18 expression were compared with Student's t-test. Statistical analyses were performed using SPSS software. A P value of less than 0.05 was considered significant.

Classes of molecules	Molecules used for treatments	Final concentrations used in treatments
Growth factors	VEGF	100 ng/ml
	TGF-β	5 ng/ml
	bFGF	20 ng/ml
Extracellular matrix proteins	Collagen I	10 μg/ml
	Fibronectin	3 μg/ml
Cytokine	TNF	200 ng/ml
NSAID	Ibuprofen	10 μM
	NS398	50 μM [45]

Table 1. The overwiev of various classes of pro-angiogenic molecules and NSAID and their concentrations used for treatments of HUVEC.

3. Results and Discussion

3.1 *SOX18* gene is expressed in HUVEC

The human *SOX18* gene is transiently expressed in nascent endothelial cells during embryonic development and postnatal angiogenesis. Therefore, we have started our study by analyzing *SOX18* gene expression in primary endothelial cells by using HUVEC, a well-established endothelial model system. In addition to RNA isolated from HUVEC, we included in this analysis RNAs isolated from HeLa cells and EA.hy926 endothelial cells. Namely, we used RT-PCR method in order to analyze the expression of *SOX18* gene in HUVEC, while HeLa and EA.hy926 cells served as positive controls, for which we have previously shown to express SOX18 [19,21]. As presented at Figure 1, after 12 cycles of amplification no products could be detected for either *SOX18* or *GAPDH* gene. Then, after 24 cycles of amplification, *SOX18* product starts to appear in HUVEC, while *GAPDH* is detectable in all tested cells at the similar level. After 35 cycles of amplification in all tested cells we detected the amplification of *SOX18* gene product and without obvious changes in *GAPDH* level between analyzed samples. Results of this analysis demonstrated that HUVEC express *SOX18* gene, and that the relative level of its expression is higher compared to HeLa or EA.hy926 cells. By this we have confirmed that these cells are an appropriate model system for further study.

3.2 The effect of pro-angiogenic growth factors on SOX18 protein level in HUVEC

Growth factors are naturally occurring proteins capable of stimulating variety of cellular processes, including cell growth, proliferation and differentiation. VEGF, bFGF (also known as FGF-2), and TGF-β play prominent and well-documented roles in angiogenesis [23,24]. Importantly, VEGF expression correlates with tumor vascularity and progression in many cancers [25]. Inhibition of VEGF results in decreased tumor vascularity and growth, implicating VEGF as the major tumor angiogenesis factor. Anti-VEGF therapies are currently used in oncology to treat metastatic cancers [26].

We have tested the effect of VEGF, bFGF and TGF-β on SOX18 protein level in HUVEC (Table 1). As presented on Figure 2A and B, treatments with bFGF for 1 h and 3 h and TGF-β for 3 h did not cause changes in the SOX18 protein level. Next, we have performed VEGF treatment of HUVEC for 0.5 h, 1 h, 3 h and 24 h and observed changes at SOX18 protein level only after 3 h treatment (Figure 2C). As presented by histogram, significant increase in SOX18 protein level of 1.7-fold

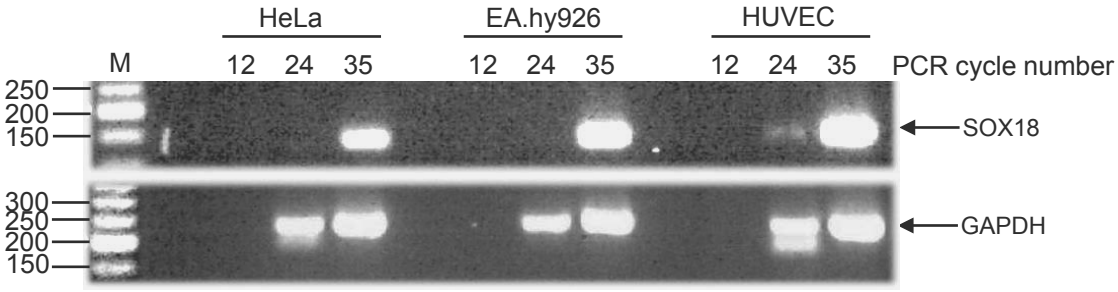

Figure 1. Analysis of *SOX18* gene expression in HUVEC by RT-PCR. *SOX18* gene expression in HeLa and EA.hy926 cells (positive controls) and in HUVEC was tested after 12, 24 and 35 amplification cycles, as indicated. GAPDH was used as internal control, M - DNA ladder.

has been detected following this treatment (Figure 2C). This finding is in concordance with data showing that approximately 45% of genes induced by VEGF are significantly up-regulated within the first 2-6 hours [27]. Furthermore, it has been shown that after stimulation of HUVEC with VEGF, *SOX18* mRNA level raises within first two hours and declines thereafter [28]. Importantly, literature data indicate that, *in vivo*, the onset of murine *Sox18* expression occurs after that of *Vegf*, around the same time as that of its receptor *Flk1* (VEGF-R2) and before that of its second receptor, *Flt1* (VEGF-R1) in mouse embryos and adult wound [10,11,29]. Thus, *Sox18* expression is placed downstream of *Vegf* indicating that this gene could be a target of VEGF growth factor.

While the increase in the *SOX18* mRNA by treatment with VEGF was demonstrated earlier [28], here, we have presented for the first time the effect of VEGF on SOX18 protein level in human endothelial setting. This observation is of functional relevance, since both factors are marked as mediators of angiogenesis, inducing proliferation, sprouting and tube formation of endothelial cells [12,14].

Among other transcription factors induced by VEGF, it has been shown that EGR1 has a peak of expression after 30 minutes of treatment and is decreased thereafter [27]. Also, VEGF treatment stimulated the accumulation of EGR1 in the nucleus within 60 minutes [30]. The functional link between VEGF and EGR1 is particularly interesting since we have recently shown

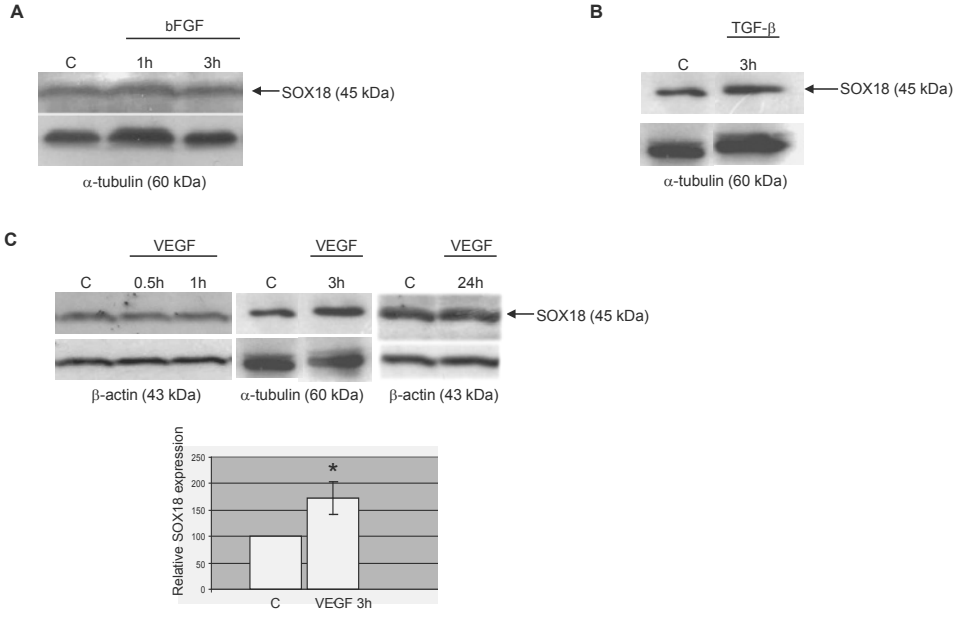

Figure 2. The effect of pro-angiogenic growth factors on SOX18 protein level in HUVEC. (A) Western blot analysis of SOX18 protein level in untreated HUVEC (lane C) and in HUVEC treated for 1 h and 3 h with bFGF, as indicated. (B) Western blot analysis of SOX18 protein level in untreated HUVEC (lane C) and in HUVEC treated for 3 h with TGF-β, as indicated. (C) Western blot analysis of SOX18 protein level in untreated HUVEC (lane C) and in HUVEC treated for 0.5 h, 1 h, 3 h and 24 h with VEGF, as indicated. The quantities of SOX18 protein in cells treated with VEGF for 3 h were calculated as a percentage of untreated cells (C), which was set as 100%. Data of at least three independent experiments are presented at histogram as the means ± SEM. Value of $P<0.05$ is represented by *.

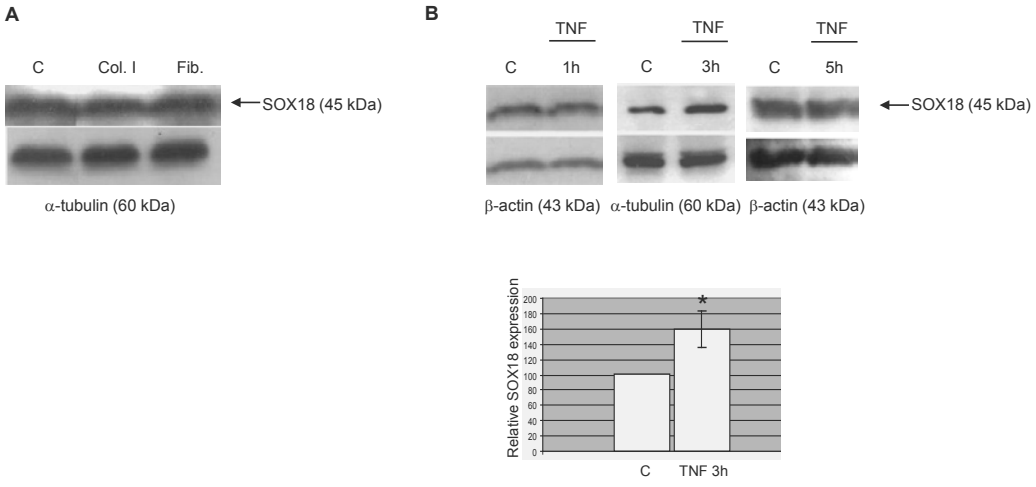

Figure 3. The effect of ECM proteins and TNF on SOX18 protein level in HUVEC. (A) Western blot analysis of SOX18 protein level in HUVEC kept in suspension (lane C) or seeded in dish pre-coated with collagen I (lane Col I) or fibronectin (lane Fib). (B) Western blot analysis of SOX18 protein level in untreated HUVEC (lane C) and in HUVEC treated for 1, 3 and 5 h with TNF, as indicated. The quantities of SOX18 protein in cells treated with TNF for 3 h were calculated as a percentage of untreated cells (C), which was set as 100%. Data of at least three independent experiments are presented at histogram as the means ± SEM. Value of $P<0.05$ is represented by *.

that *SOX18* promoter activity is positively regulated by EGR1 [21]. Thus, it would be interesting to investigate whether EGR1 mediates the *SOX18* expression elicited by VEGF, thereby giving a novel insight into possible functional relation between EGR1 and SOX18 during angiogenesis.

3.3 The effect of extracellular matrix proteins on SOX18 protein level in HUVEC

Endothelial cell adhesion and migration are important events during development, wound healing and angiogenesis [31]. The capacity of endothelial cells to migrate depends on the interaction between ECM proteins and cell surface integrins, which subsequently initiate intracellular signaling pathways responsible for endothelial cell spreading and migration [32].

We have therefore tested whether seeding of HUVEC in dishes pre-coated with ECM proteins could affect *SOX18* expression compared to non-adherent HUVEC. For that purpose we have used collagen I and fibronectin (Table 1), two ECM proteins binding to integrin $\alpha_2\beta_1$ and $\alpha_5\beta_1/\alpha_V\beta_3$, respectively. Integrins $\alpha_2\beta_1$ and $\alpha_V\beta_3$ mediate *in vitro* endothelial cell attachment, spreading, and migration [33], and are transiently localized on endothelial cells at the tips of capillary sprouts during wound repair *in vivo* [34]. Our results showed that none of the tested ECM proteins had effect on SOX18 protein level (Figure 3A), suggesting that ligation and activation of $\alpha_2\beta_1$, $\alpha_5\beta_1$ and $\alpha_V\beta_3$ integrins does not lead to alteration of SOX18 expression in HUVEC.

3.4 The effect of TNF on SOX18 protein level in HUVEC

Tumor necrosis factor, a potent cytokine predominantly produced in macrophages, is able to induce cell death, inflammation, and to inhibit tumorigenesis and viral replication [35,36]. Also, it has been shown that TNF stimulates angiogenesis *in vivo* [37] and formation of endothelial cell tubes *in vitro* [38]. We analyzed whether treatments of HUVEC with this cytokine for 1 h, 3 h and 5 h could affect SOX18 protein level. As presented in Figure 3B, only treatment for 3 h caused change in SOX18 protein level, while 1 h and 5 h treatments had no effect. As presented by histogram, significant increase in SOX18 protein level of 1.6-fold has been detected following 3 h treatment (Figure 3B).

TNF-induced survival involves the activation of the transcription factor NF-κB [39]. We have performed *in silico* analysis and detected several putative NF-κB binding sites within the promoter of human *SOX18* gene (data not shown) that could be of functional relevance for its induction by TNF. Further, it has been shown earlier that TNF has the ability to inhibit Sp1 and Sp3 binding to their consensus sites [40]. Since we have shown previously that transcription factor Sp3 acts as a repressor of *SOX18* promoter activity [20], we hypothesize that TNF induced SOX18 expression is, at least in part, associated with inhibited binding of Sp3. In addition, the expression of transcription factor EGR1, shown to be able to up-regulate *SOX18* promoter activity [21], is also stimulated by TNF [41].

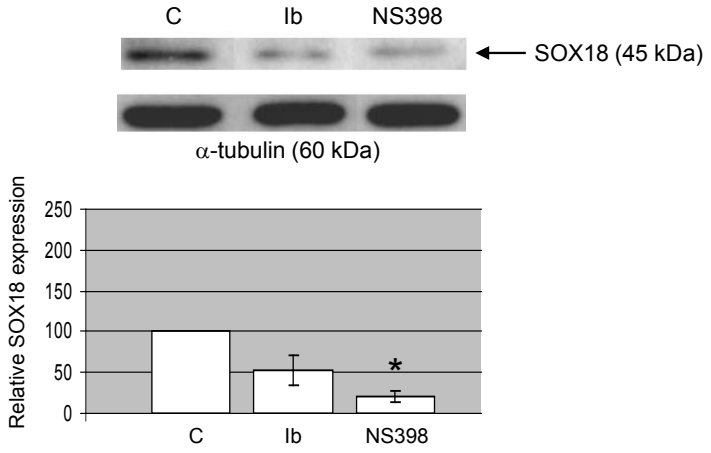

Figure 4. The effect of NSAID on SOX18 protein level in HUVEC. Western blot analysis of SOX18 protein level in untreated HUVEC (lane C) and in HUVEC treated for 3 h with ibuprofen (lane Ib) and NS398 (lane NS398). The quantities of SOX18 protein in cells were calculated as a percentage of untreated cells (C), which was set as 100%. Data of at least two independent experiments are presented at histogram as the means ± SEM. Value of $P<0.05$ is represented by *.

3.5 The effect of NSAID on SOX18 protein level in HUVEC

NSAID inhibit the two isoforms of cyclooxygenase, COX-1 and COX-2, the rate-limiting enzymes on the prostanglandins biosynthetic pathway. Prostanglandins are potent pro-inflammatory molecules also able to stimulate angiogenesis *in vivo* and *in vitro* [42-44]. Thus, NSAID, among other effects, also act as anti-angiogenic drugs. In this study we have used ibuprofen, as a nonspecific COX-1/2 inhibitor, and NS398, as a specific COX-2 inhibitor, to test the effect of COX inhibition on SOX18 protein expression in HUVEC. The obtained results demonstrate that selected NSAID are capable of down regulating the level of SOX18 protein in endothelial cells. In particular, after treatment of HUVEC with ibuprofen and NS398 for three hours, SOX18 protein level was decreased to approximately 50% and 25%, respectively, in comparison to control HUVEC (Figure 4).

Regardless of the fact that NSAID act as pleiotropic agents, the demonstration that they can reduce SOX18 expression in endothelial cells, opens the possibility for pharmacological modulation of this gene expression. More importantly, we believe that finding targets of COX inhibitors that mediate some of their anti-angiogenic effects will help in selecting molecules, such as SOX18, that would become targets for other, more selective anti-angiogenic agents.

However, the mechanisms by which NSAID inhibit angiogenesis are likely to be multifactorial, including inhibition of mitogen-activated protein (Erk2) kinase; suppression of cell cycle proteins; inhibition of EGR1 gene activation; interference with hypoxia inducible factor 1 and VEGF gene activation; increased production of endogenous angiogenesis inhibitor; inhibition of endothelial cell proliferation, migration and spreading; and induction of endothelial apoptosis [45,46]. Since it has been shown that NSAID inhibit the activation of *egr-1* gene in microvascular endothelial cells [47], we hypothesize that this inhibition may be functionally relevant for the down regulation of SOX18 expression.

In summary, in order to study the involvement of human *SOX18* gene in signaling events that promote angiogenesis, we have demonstrated that the angiogenic factor VEGF and the pro-inflammatory cytokine TNF increase SOX18 protein level, while pan-COX-1/2 and COX-2-specific inhibitors (*i.e.* ibuprofen and NS398, respectively) decrease SOX18 protein level in endothelial cells. These data represent a relevant contribution to the understanding of the involvement of SOX18 in the angiogenic processes. However, this manuscript represents our initial effort in understanding the extracellular cues and intracellular signaling pathways involved in the regulation of SOX18 expression. It will serve as a basis for the future, more comprehensive work that would lead to delineation of the exact role of SOX18 in signaling events important for the angiogenic switch of endothelial cells.

Transcription factors are modulated by signaling events elicited by extracellular factors and cell surface receptors and in turn, they regulate the expression of the effector molecules in signaling cascades. In this perspective, it has been proposed that targeting particular transcription factor can affect biological process more profoundly than targeting individual upstream factor, receptor, signaling molecule or downstream effector [18]. In recent studies, *Sox18* gene, expressed during initial steps of tumor vascularization,

has been recognized as a potential target for anti-angiogenic therapy [12]. Since tumor growth is dependent on a blood supply, anti-angiogenic therapy of cancer represents a potentially highly effective strategy for restraining tumors [48]. Thus, clarifying the complex mechanisms involved in the regulation of human *SOX18* gene expression will help in better understanding of important physiological and patho- physiological processes in which this transcription factor participates.

Acknowledgements

This work has been supported by grants from Ministry of Science and Technological Development, Republic of Serbia (Grant No. 143028), and from the International Centre for Genetic Engineering and Biotechnology (Grant No. CRP/YUG 07-01) to I.P., G.N. and M.S., and by Grant from the Medic Foundation to J.Z. and C.R.

References

[1] Folkman J., Shing Y., Angiogenesis, J. Biol. Chem., 1992, 267, 10931-10934
[2] Folkman J., D'Amore P.A., Blood vessel formation: what is its molecular basis?, Cell, 1996, 87, 1153-1155
[3] Risau W., Mechanisms of angiogenesis, Nature, 1997, 386, 671-674
[4] Felmeden D.C., Blann A.D., Lip G.Y., Angiogenesis: basic pathophysiology and implications for disease, Eur. Heart J., 2003, 24, 586-603
[5] Folkman J., Watson K., Ingber D., Hanahan D., Induction of angiogenesis during the transition from hyperplasia to neoplasia, Nature, 1989, 339, 58-61
[6] Hanahan D., Folkman J., Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis, Cell, 1996, 86, 353-364
[7] Pevny L.H., Lovell-Badge R., Sox genes find their feet, Curr. Opin. Genet. Dev., 1997, 7, 338-344
[8] Wegner M., From head to toes: the multiple facets of Sox proteins, Nucl. Acids Res., 1999, 27, 1409-1420
[9] Downes M., Koopman P., SOX18 and the transcriptional regulation of blood vessel development, Trends Cardiovasc. Med., 2001, 11, 318-324
[10] Pennisi D., Gardner J., Chambers D., Hosking B., Peters J., Muscat G., et al., Mutations in Sox18 underlie cardiovascular and hair follicle defects in ragged mice, Nat. Genet., 2000, 24, 434-437
[11] Darby I.A., Bisucci T., Raghoenath S., Olsson J., Muscat G.E., Koopman P., Sox18 is transiently expressed during angiogenesis in granulation tissue of skin wounds with an identical expression pattern to Flk-1 mRNA, Lab Invest., 2001, 81, 937-943
[12] Young N., Hahn C.N., Poh A., Dong C., Wilhelm D., Olsson J., et al., Effect of disrupted SOX18 transcription factor function on tumor growth, vascularization, and endothelial development, J. Natl. Cancer Inst., 2006, 98, 1060-1067
[13] Irrthum A., Devriendt K., Chitayat D., Matthijs G., Glade C., Steijlen P.M., et al., Mutations in the transcription factor gene SOX18 underlie recessive and dominant forms of hypotrichosis-lymphedema-telangiectasia, Am. J. Hum. Genet., 2003, 72, 1470-1478
[14] Otrock Z.K., Mahfouz R.A., Makarem J.A., Shamseddine A.I., Understanding the biology of angiogenesis: review of the most important molecular mechanisms, Blood Cells Mol. Dis., 2007, 39, 212-220
[15] Ferrara N., VEGF as a therapeutic target in cancer, Oncology, 2005, 69 Suppl. 3, 11-16
[16] Schneider B.P., Sledge G.W., Jr., Drug insight: VEGF as a therapeutic target for breast cancer, Nat. Clin. Pract. Oncol., 2007, 4, 181-189
[17] Sato Y., Molecular mechanism of angiogenesis transcription factors and their therapeutic relevance, Pharmacol. Ther., 2000, 87, 51-60
[18] Xie K., Wei D., Huang S., Transcriptional anti-angiogenesis therapy of human pancreatic cancer, Cytokine Growth Factor Rev., 2006, 17, 147-156
[19] Petrovic I., Stevanovic M., The human SOX18 gene: expression analysis and characterization of its 5' flanking region, Arch. Biol. Sci., 2007, 59, 267-272
[20] Petrovic I., Kovacevic-Grujicic N., Stevanovic M., ZBP-89 and Sp3 down-regulate while NF-Y up-regulates SOX18 promoter activity in HeLa cells, Mol. Biol. Rep., 2009, 36, 993-1000
[21] Petrovic I., Grujicic N.K., Stevanovic M., Early Growth Response Protein 1 Acts as an Activator of Sox18 Promoter, Exp. Mol. Med., 2010, 42, 132-142
[22] Ruegg C., Yilmaz A., Bieler G., Bamat J., Chaubert P., Lejeune F.J., Evidence for the involvement of endothelial cell integrin alphaVbeta3 in the disruption of the tumor vasculature induced by TNF and IFN-gamma, Nat. Med., 1998, 4, 408-414
[23] Presta M., Dell'Era P., Mitola S., Moroni E., Ronca R., Rusnati M., Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis, Cytokine Growth Factor Rev., 2005, 16, 159-178

[24] Massague J., Blain S.W., Lo R.S., TGFbeta signaling in growth control, cancer, and heritable disorders, Cell, 2000, 103, 295-309
[25] Ferrara N., Vascular endothelial growth factor: basic science and clinical progress, Endocr. Rev., 2004, 25, 581-611
[26] Ferrara N., Hillan K.J., Gerber H.P., Novotny W., Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer, Nat. Rev. Drug Discov., 2004, 3, 391-400
[27] Abe M., Sato Y., cDNA microarray analysis of the gene expression profile of VEGF-activated human umbilical vein endothelial cells, Angiogenesis, 2001, 4, 289-298
[28] Garcia-Ramirez M., Martinez-Gonzalez J., Juan-Babot J.O., Rodriguez C., Badimon L., Transcription factor SOX18 is expressed in human coronary atherosclerotic lesions and regulates DNA synthesis and vascular cell growth, Arterioscler. Thromb. Vasc. Biol., 2005, 25, 2398-2403
[29] Dumont D.J., Fong G.H., Puri M.C., Gradwohl G., Alitalo K., Breitman M.L., Vascularization of the mouse embryo: a study of flk-1, tek, tie, and vascular endothelial growth factor expression during development, Dev. Dyn., 1995, 203, 80-92
[30] Sassa Y., Hata Y., Murata T., Yamanaka I., Honda M., Hisatomi T., et al., Functional role of Egr-1 mediating VEGF-induced tissue factor expression in the retinal capillary endothelium, Graefes Arch. Clin. Exp. Ophthalmol., 2002, 240, 1003-1010
[31] Lamalice L., Le Boeuf F., Huot J., Endothelial cell migration during angiogenesis, Circ. Res., 2007, 100, 782-794
[32] Clark E.A., Brugge J.S., Integrins and signal transduction pathways: the road taken, Science, 1995, 268, 233-239
[33] Leavesley D.I., Schwartz M.A., Rosenfeld M., Cheresh D.A., Integrin beta 1- and beta 3-mediated endothelial cell migration is triggered through distinct signaling mechanisms, J. Cell Biol., 1993, 121, 163-170
[34] Clark R.A., Tonnesen M.G., Gailit J., Cheresh D.A., Transient functional expression of alphaVbeta 3 on vascular cells during wound repair, Am. J. Pathol., 1996, 148, 1407-1421
[35] Williams G.M., Antitumor necrosis factor-alpha therapy and potential cancer inhibition, Eur. J. Cancer Prev., 2008, 17, 169-177
[36] Zhao X., Bausano B., Pike B.R., Newcomb-Fernandez J.K., Wang K.K., Shohami E., et al., TNF-alpha stimulates caspase-3 activation and apoptotic cell death in primary septo-hippocampal cultures, J. Neurosci. Res., 2001, 64, 121-131
[37] Frater-Schroder M., Risau W., Hallmann R., Gautschi P., Bohlen P., Tumor necrosis factor type alpha, a potent inhibitor of endothelial cell growth in vitro, is angiogenic in vivo, Proc. Natl. Acad. Sci. USA, 1987, 84, 5277-5281
[38] Leibovich S.J., Polverini P.J., Shepard H.M., Wiseman D.M., Shively V., Nuseir N., Macrophage-induced angiogenesis is mediated by tumour necrosis factor-alpha, Nature, 1987, 329, 630-632
[39] Hayden M.S., Ghosh S., Signaling to NF-kappaB, Genes Dev., 2004, 18, 2195-2224
[40] Denson L.A., Menon R.K., Shaufl A., Bajwa H.S., Williams C.R., Karpen S.J., TNF-alpha downregulates murine hepatic growth hormone receptor expression by inhibiting Sp1 and Sp3 binding, J. Clin. Invest., 2001, 107, 1451-1458
[41] Goetze S., Kintscher U., Kaneshiro K., Meehan W.P., Collins A., Fleck E., et al., TNFalpha induces expression of transcription factors c-fos, Egr-1, and Ets-1 in vascular lesions through extracellular signal-regulated kinases 1/2, Atherosclerosis, 2001, 159, 93-101
[42] Dubois R.N., Abramson S.B., Crofford L., Gupta R.A., Simon L.S., Van De Putte L.B., et al., Cyclooxygenase in biology and disease, Faseb J, 1998, 12, 1063-1073
[43] Form D.M., Auerbach R., PGE2 and angiogenesis, Proc. Soc. Exp. Biol. Med., 1983, 172, 214-218
[44] Cheng T., Cao W., Wen R., Steinberg R.H., LaVail M.M., Prostaglandin E2 induces vascular endothelial growth factor and basic fibroblast growth factor mRNA expression in cultured rat Muller cells, Invest. Ophthalmol. Vis. Sci., 1998, 39, 581-591
[45] Tarnawski A.S., Jones M.K., Inhibition of angiogenesis by NSAIDs: molecular mechanisms and clinical implications, J. Mol. Med., 2003, 81, 627-636
[46] Dormond O., Foletti A., Paroz C., Ruegg C., NSAIDs inhibit alpha V beta 3 integrin-mediated and Cdc42/Rac-dependent endothelial-cell spreading, migration and angiogenesis, Nat. Med., 2001, 7, 1041-1047
[47] Szabo I.L., Pai R., Soreghan B., Jones M.K., Baatar D., Kawanaka H., et al., NSAIDs inhibit the activation of egr-1 gene in microvascular endothelial cells. A key to inhibition of angiogenesis?, J. Physiol. Paris, 2001, 95, 379-383
[48] Feldman A.L., Libutti S.K., Progress in antiangiogenic gene therapy of cancer, Cancer, 2000, 89, 1181-1194