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Early growth response protein 1 acts as an activator of *SOX18* promoter

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Abbreviations: CAT, chloramphenicol acetyltransferase; EGR1, early growth response 1; EMSA, electrophoretic mobility shift assay; HMG, high mobility group box; NF-Y, nuclear factor Y; SOX, sex-determining region Y box; Sp3, specificity protein 3; *tsp*, transcription start point; WCL, whole cell lysates; ZBP-89, zinc finger binding protein 89

Abstract

Sex-determining region Y box 18 (*Sox18/SOX18*) gene is an important regulator of vascular development playing a role in endothelial cell specification or differentiation, angiogenesis and atherogenesis. The aim of this study was to perform comprehensive functional characterization of the human *SOX18* promoter, including determination of transcription start point (*tsp*) and identification of control elements involved in the regulation of *SOX18* gene expression, with an emphasis on angiogenesis-related transcription factors. Analyses were performed in HeLa cells, representing a tumor cell line, and in EA.hy926 cells used as an endothelial model system. We have determined unique *tsp* of *SOX18* gene, located 172 nucleotides upstream from ATG codon. Further, we have shown that *SOX18* promoter region, -726 to -89 bp relative to *tsp*, contains positive *cis*-regulatory element(s) that stimulates *SOX18* promoter activity, while region -89 to + 166 represents the minimal promoter. Within this region we have recognized the presence of essential element(s), positioned from -89 to +29, which harbors cluster of three putative early growth response 1 (EGR1) binding sites. By *in vitro* binding assays and functional analyses we have shown that these three putative binding sites are functionally relevant and sufficient for EGR1-induced *SOX18* transcription. Mutations of these binding sites significantly impaired activity of the *SOX18* promoter, particularly in EA.hy926 cells, indicating the importance of these regulatory elements for *SOX18* promoter activity in endothelial setting. By data presented in this study, we have established *SOX18* as a novel target gene regulated by EGR1 transcription factor, thus providing the first functional link between two transcription factors previously shown to be involved in the control of angiogenesis.

Keywords: early growth response protein 1; endothelial cells; neovascularization, physiologic; promoter regions, genetic; SOX18 protein, human; transcription, genetic

Introduction

Sex-determining region Y box (*SOX*) genes constitute a large family of diverse and well-conserved genes encoding transcription factors implicated in the control of various developmental processes (Pevny and Lovell-Badge, 1997; Wegner, 1999). *SOX* proteins contain a characteristic DNA-binding motif commonly known as the high mobility group domain (HMG domain) and display properties of both classical transcription factors and architectural components of chromatin (Pevny and Lovell-Badge, 1997). Based on homologies, both within and outside of the HMG box, this gene family can be subdivided into ten groups, A to J (Bowles *et al.,* 2000). *Sox18/SOX18* gene, together with *Sox7/SOX7* and *Sox17/SOX17* genes, belongs to *SOX* group F. Previously, it has been shown that *Sox* group F genes have a role in vascular development and postnatal neovascularization (Matsui *et al.,* 2006; Cermenati *et al.,* 2008). The functional importance of *SOX18* protein in vascular development is revealed by the vascular defects caused by *Sox18/SOX18* mutations in mice and humans. Mutations in *Sox18* underlie the mutant phenotype of *ragged* mutant mouse (Downes and Koopman, 2001) and mutations in human *SOX18* are associated with hypotrichosis-lymphedema-telangiectasia syndrome (Irrthum *et al.,* 2003). Murine *Sox18* is demonstrated to be involved in the induction of angiogenesis during wound healing and tissue repair (Darby *et al.,* 2001) and *SOX18* is shown to play a role in atherosclerosis in humans (Garcia-

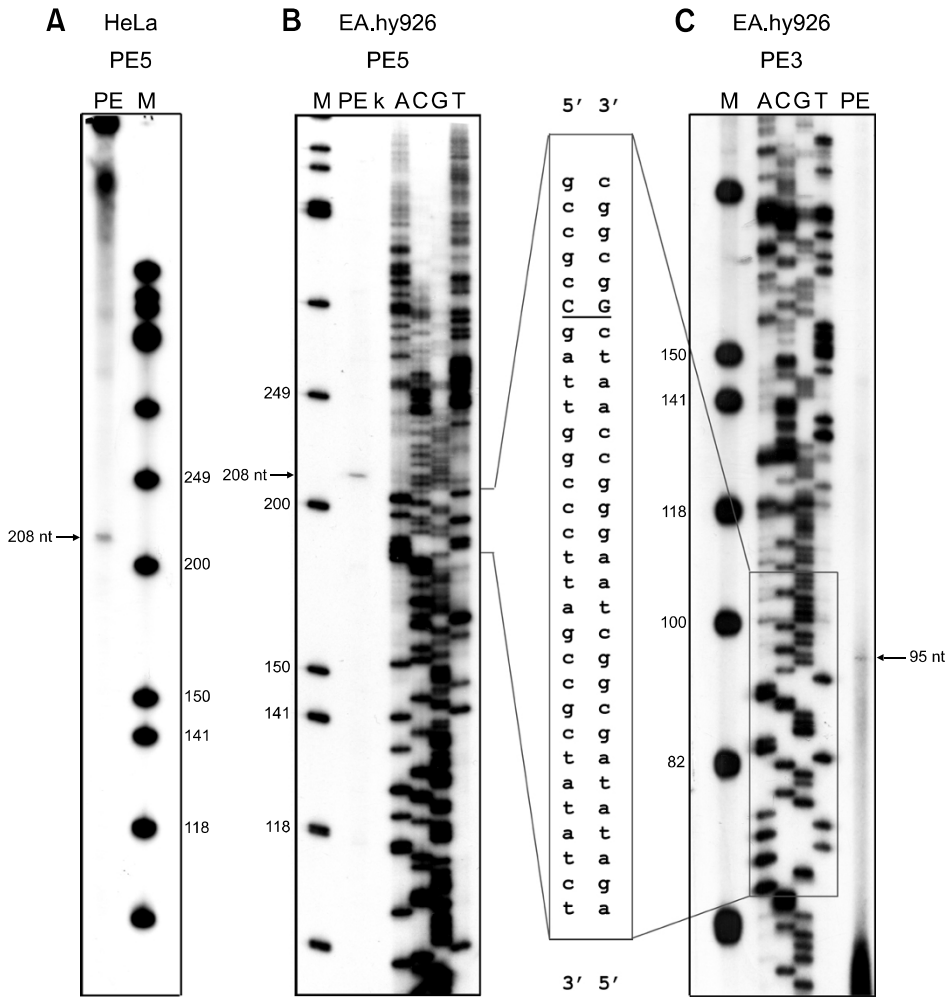

Figure 1. Mapping of the *SOX18* tsp by primer extension analysis. Total RNA from HeLa (A) and EA.hy926 cells (B, C) were hybridized to the radiolabeled antisense primers PE5 (A, B) and PE3 (C), and primer extension reactions were performed as described in Methods. Control reaction was performed with yeast tRNA (B, lane k). The extension products are marked by arrows (A, B, C, lanes PE). The radiolabeled *PhiX/Hin*fIII digest was used as a ladder (A, B, C, lane M). Lanes ACGT indicate the nucleotide sequence generated with the corresponding primers. The DNA sequence is shown with first nucleotide of the transcript underlined and presented in capital case.

Ramirez *et al.,* 2005). Furthermore, it has been demonstrated that interfering with *SOX18* function inhibits blood vessel formation and subsequent tumor growth (Young *et al.,* 2006).

Despite the mounting evidence that *SOX18* protein is an important player in vascular development, atherogenesis and angiogenesis, little is known about transcription factors involved in tissue and cell-type specific regulation of its expression. So far, we have characterized the *SOX18* promoter region and demonstrated that ubiquitous transcription factors specificity protein 3 (Sp3), zinc finger binding protein 89 (ZBP-89) and nuclear factor Y (NF-Y) are involved in the regulation of its expression in HeLa tumor cell line (Petrovic and Stevanovic, 2007; Petrovic *et al.,* 2009). Here, we have been particularly interested in discovering angiogenesis-related transcription factor(s) that might have the role in regulation of *SOX18* gene expression in endothelial setting.

It has been shown that early growth response protein 1 (EGR1) plays a pivotal role in the transcriptional response of endothelial cells to angiogenic growth factors involved in angiogenic switch (Lucerna *et al.,* 2006). Namely, EGR1, a zinc-finger transcription factor that binds to GC-rich *cis*-acting promoter elements controls the expression of a wide variety of pathogenesis-relevant genes, including growth factors, cytokines and receptors, with many of them involved in angiogenesis and tumorigenesis (Fahmy *et al.,* 2003; Lucerna *et al.,* 2003). There is a number of genes identified as possible EGR1 targets and they play functional roles in normal development and differentiation, as well as, in the various pathological settings (Silverman and Collins, 1999).

In this report, we have presented data demonstrating transcriptional up-regulation of the human *SOX18* gene by EGR1 in tumor (HeLa) and endothelial (EA.hy926) cells. EA.hy926 is a permanent human cell line derived from human umbilical vein endothelial cell (HUVEC) that displays a number of

features characteristic for vascular endothelial cells (Edgell *et al.,* 1983). Therefore, these cells were used as an endothelial model system in the present study.

Presented results provide the first functional link between *SOX18* and EGR1, previously shown to be involved in the regulation of angiogenesis. Understanding of molecular mechanisms involved in human *SOX18* gene transcriptional regulation could help in better understanding of important physiological and patho-physiological processes in which *SOX18* participates.

Results

Mapping of the *SOX18* transcription start point

The first step in functional characterization of *SOX18* promoter was to determine the tsp of the human *SOX18* gene. For that purpose we have performed primer extension analysis, using total RNA isolated from HeLa and EA.hy926 cells. Single products were obtained with the length of 208 nucleotides and 95 nucleotides with PE5 and PE3 primers, respectively (Figures 1A, 1B and 1C). Comparison to dideoxy-sequencing reaction revealed that both primers extended to the same cytosine residue, 172 nucleotides upstream from ATG codon and this nucleotide was designated as +1 (Figures 1B and 1C). These results demonstrate that *SOX18* transcription initiates at the single major site, regardless of different origin of HeLa and EA.hy926 cells. The position of *tsp*, determined by this analysis, correlates with the length of *SOX18* transcript that we have detected earlier by Northern blotting (Petrovic and Stevanovic, 2007).

Transcriptional activity of *SOX18* promoter deletion constructs

Previously, we demonstrated that *SOX18* 5' flanking fragment 892 bp in size upstream from the start codon represents *SOX18* promoter region (Petrovic and Stevanovic, 2007). Here, the same regulatory region, together with its three different deletion fragments, has been subjected to further functional analysis in HeLa and EA.hy926 cells (Figure 2A). As presented on Figure 2B, the full length promoter construct (892pCAT6) displayed maximum reporter activity, while 5' deletion up to -89 (255pCAT6) caused decrease of CAT activity to approximately 30% and 60% in HeLa and EA.hy926 cells, respectively. These results indicate that *SOX18* promoter region, spanning the sequence -726 to -89 bp relative to *tsp*, contains positive *cis*-regulatory element(s) responsible for

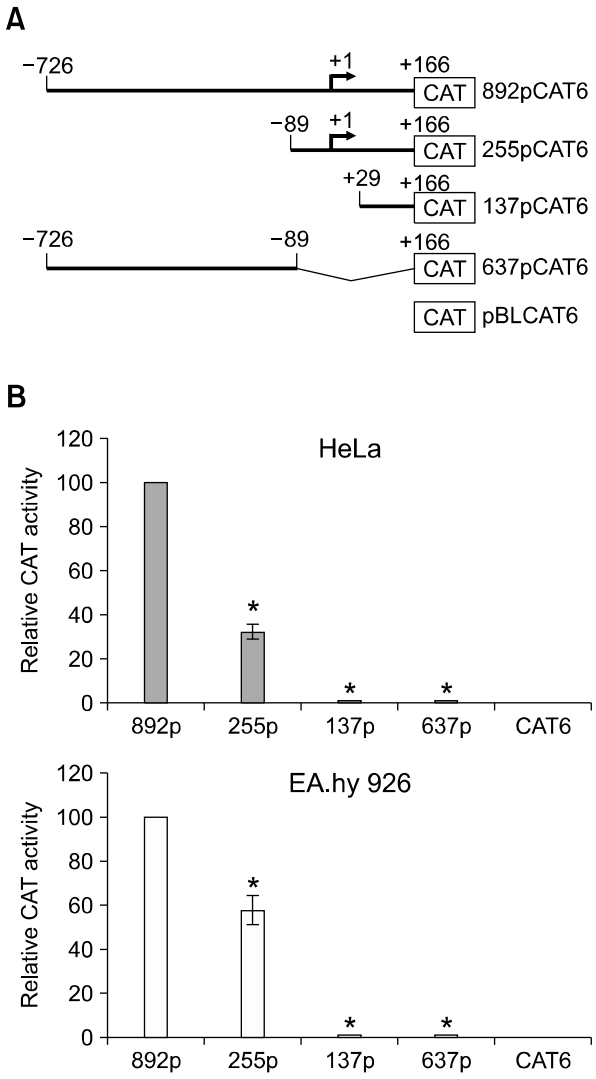

Figure 2. Functional analysis of the human *SOX18* promoter. (A) Schematic representation of the cat reporter constructs. Numbers indicate the positions of fragments end points relative to the *tsp.* (B) Reporter activities of the *SOX18* promoter constructs in HeLa cells (grey bars) and EA.hy926 (white bars). The normalized CAT activities were evaluated as a percentage of the 892pCAT6 construct, which was set as 100% and are presented as the means ± SEM of at least five independent experiments. Values of $P < 0.001$ are presented by *.

stimulation of *SOX18* promoter activity. Further 5' deletion up to +29 (construct 137pCAT6) resulted in complete abolition of the promoter activity, thus revealing the presence of essential element(s) positioned between -89 and +29 relative to *tsp* (Figure 2B). Additionally, 3' deletion of the full length construct up to -89 (construct 637pCAT6) caused complete loss of promoter activity, indicating that region -89 to +166 bp relative to *tsp* (construct 255pCAT6) harbors regulatory elements indispensable for *SOX18* transcription in both cell lines and represents the minimal promoter region

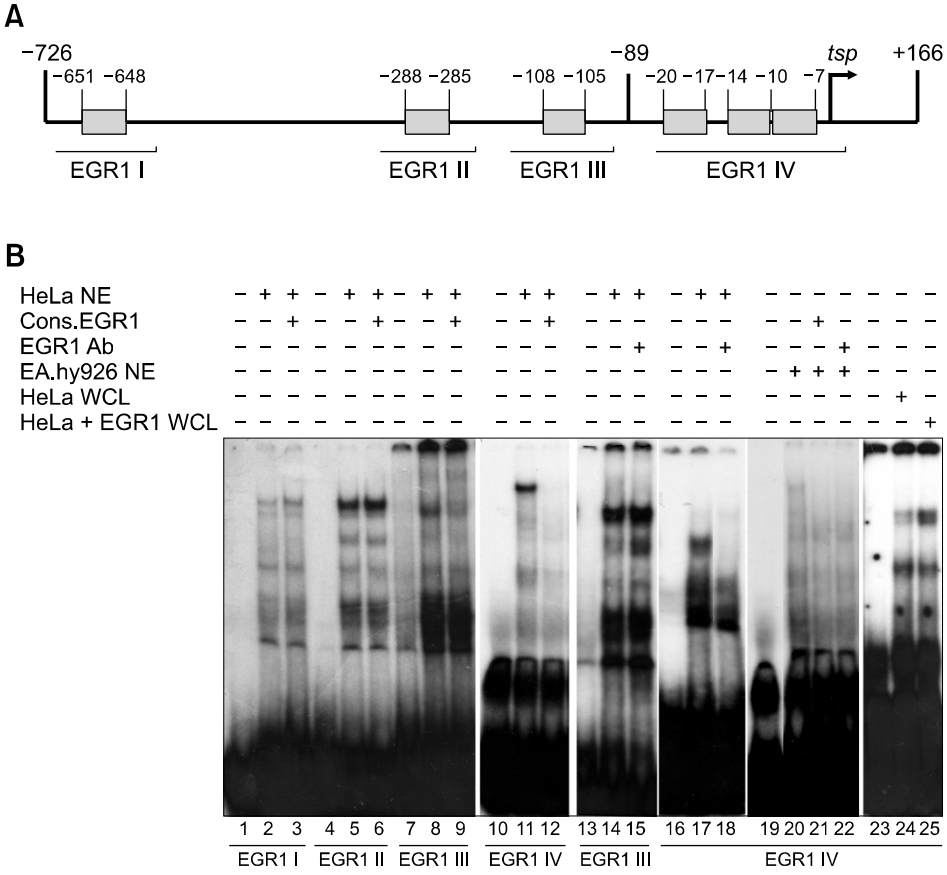

Figure 3. EMSA with oligonucleotide probes containing putative EGR1 binding sites. (A) Schematic illustration of the *SOX18* promoter with delineated putative EGR1 binding sites. The positions of putative EGR1 core binding sites, relative to *tsp*, are indicated above and relative positions of corresponding probes are presented by dotted lines. (B) EMSA of the corresponding EGR1 probes with HeLa nuclear extracts (HeLa NE, lanes 2, 5, 8 ,11, 14 and 17) and EAhy.926 nuclear extracts (EA.hy926 NE, lane 20). Competition assays with 100-fold molar excess of unlabeled consensus EGR1 oligonucleotide (lanes 3, 6, 9, 12 and 21). Supershift assay with anti-EGR1 Ab (lanes 15, 18 and 22). EMSA with WCL prepared from either, mock transfected HeLa cells (lane 24) or cells transfected with pcDNA3.1-EGR1 expression vector (lane 25).

of the *SOX18* gene.

EGR1 binds within *SOX18* minimal promoter region *in vitro*

Using *in silico* analysis, within *SOX18* promoter region, we have identified a plethora of putative binding sites for various, mainly ubiquitous transcription factors (data not shown). Among them, we have identified six putative binding sites for angiogenesis-related, zing-finger transcription factor EGR1 (Figure 3A). As shown on Figure 3A, three putative EGR1 binding sites are located within the region -726 to -89 bp relative to *tsp*, and another three are clustered within minimal promoter region immediately upstream of the *tsp*. In order to determine to which of these putative binding sites EGR1 binds, we have performed EMSA with four *SOX18* DNA probes that harbor different putative EGR1 binding sites (designated as probes EGR1 I to IV, Figure 3A). Nuclear proteins isolated from HeLa cells bind to all four DNA probes and form DNA-protein complexes (Figure 3B, lanes 2, 5, 8 and 11). In competition reaction, EGR1 consensus oligonucleotide probe was used in order to elucidate whether EGR1 participates in DNA-protein complex formation. In competition reaction with 100-fold molar excess of unlabeled EGR1 consensus probe, we observed complete fading of DNA-protein complexes formed by EGR1 IV probe (Figure 3B, lane 12), moderate competition with EGR1 III probe (Figure 3B, lane 9), and no competition with other two probes (Figures 3B, lanes 3 and 6). Accordingly, supershift assays were performed with probes EGR1 III and EGR1 IV only. After addition of anti-EGR1 antibody no supershift or fading of complexes were observed with EGR1 III probe (Figure 3B, lane 15), while significant fading of slowest DNA-protein migrating complex was observed with EGR1 IV probe (Figure 3B, lane 18). Therefore, we extended our analyses with EGR1 IV probe, using nuclear proteins from EA.hy926 cells (Figure 3B, lanes 19 to 22). Both competition with EGR1 consensus probe and addition of anti-EGR1 antibody caused fading of DNA-protein complexes (Figure 3B, lanes 21 and 22, respectively). Fading of DNA-protein complexes in supershift reaction has already been shown in several reports and was considered as a confirmation of specific protein presence (Argyropoulos *et al.,* 2003; Kovacevic Grujicic *et al.,* 2005). In order to further validate obtained results, we have per-

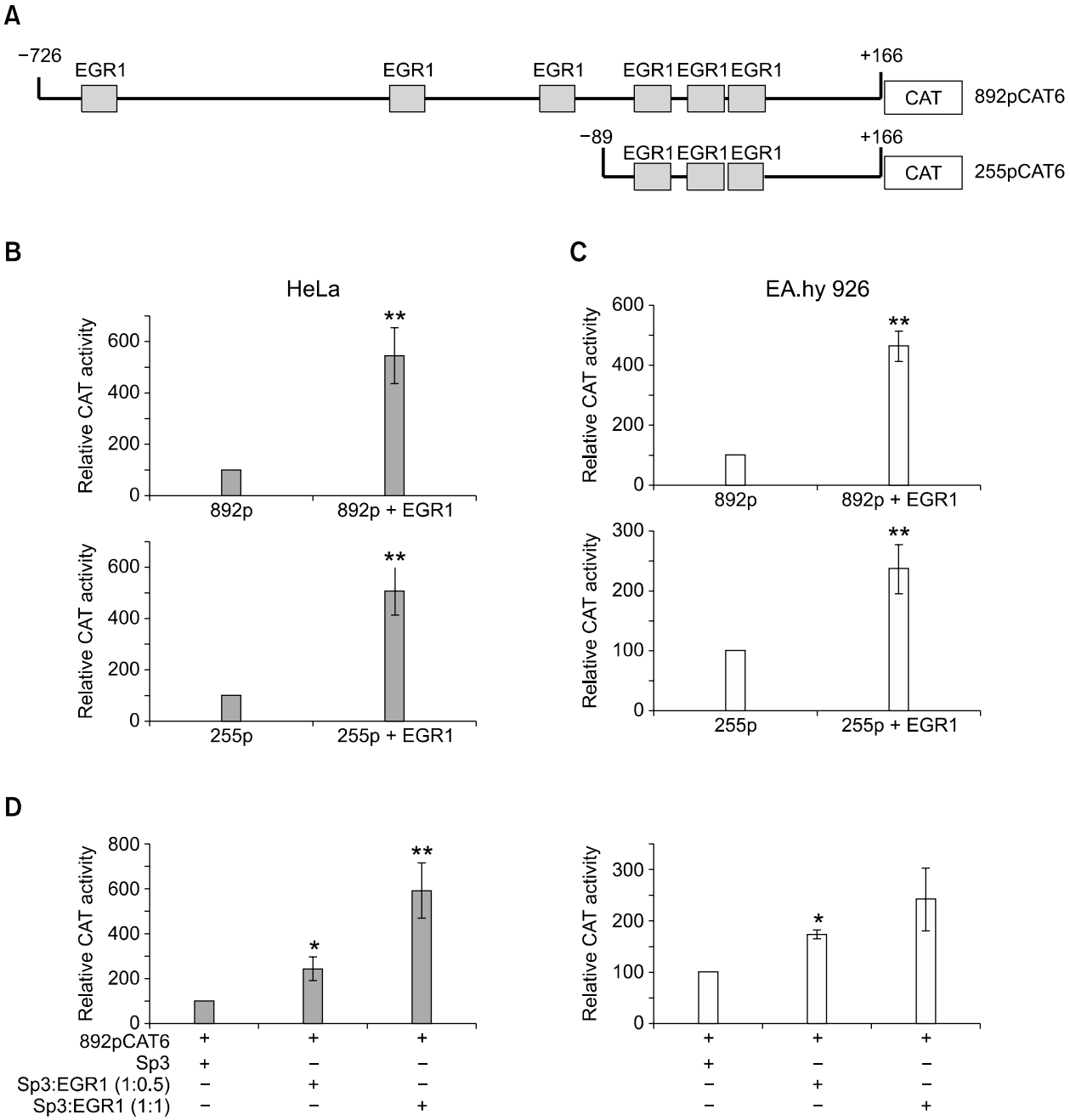

Figure 4. Effects of EGR1 overexpression on *SOX18* promoter activity. (A) Schematic illustration of the 892pCAT6 and 255pCAT6 promoter reporter constructs. Grey boxes represent putative EGR1 binding sites within *SOX18* promoter region. HeLa cells (B) and EA.hy926 cells (C) were transiently co-transfected with either 892pCAT6 or 255pCAT6 promoter constructs, together with either empty or EGR1 expression vector. Normalized CAT activities were calculated as percentages of the corresponding reporter construct activity in cells co-transfected with empty pcDNA3.1 (which was set as 100%). Relative CAT activities are presented as the means ± SEM of three independent experiments. Values of $P < 0.05$ are presented by**. (D) Functional competition between Sp3 and EGR1 performed with 892pCAT6 promoter construct. HeLa and EA.hy926 cells were co-transfected with fixed amount of Sp3 expression vector and increasing amounts of EGR1 expression vector, as indicated. Normalized CAT activities were calculated as percentages of the corresponding reporter construct activity in cells co-transfected with Sp3 (which was set as 100%). Relative CAT activities are presented as the means ± SEM of at least three independent experiments. Values of $P < 0.001$ are presented by* and $P < 0.05$ are presented by**.

formed EMSA reaction with proteins isolated from HeLa cells transfected with EGR1 expression vector. Compared to the binding of proteins isolated from mock transfected HeLa cells, we have detected increased binding of proteins when exogenous EGR1 is overexpressed (Figure 3B, lanes 24 and

25). This result demonstrated that EGR1 overexpression significantly increased cellular protein binding to specific *SOX18* oligonucleotide probe, indicating its participation in DNA-protein complex formation.

Taken together, we have shown that EGR1 transcription factor specifically binds to the sequence -29 to +10 relative to *tsp* (EGR1 IV probe), within *SOX18* minimal promoter region.

EGR1 up-regulates *SOX18* promoter activity in Hela and EA.hy926 cell lines

In order to explore the role of EGR1 in transcriptional regulation of the human *SOX18* gene expression, we have performed transient co-transfection experiments of HeLa and EA.hy926 cells with EGR1 expression vector. We have tested the effect of EGR1 overexpression on the activities of both, full length promoter (construct 892pCAT6) and minimal promoter region (construct 255pCAT6) (Figure 4A). Full length promoter construct (892pCAT6) activity was increased approximately 5-fold in both HeLa and EA.hy926 cells (Figures 4B and 4C). Interestingly, response of minimal promoter construct (255pCAT6) to EGR1 overexpression was different between two tested cell lines. Precisely, 255pCAT6 activity was increased approximately 5-fold in HeLa and 2.5-fold in EA.hy926 cells (Figures 4B and 4C).

Thus, in HeLa cell line, EGR1 overexpression up-regulated both promoter constructs to the same extent (Figure 4B). On the other hand, in EA.hy926 cells 2-fold attenuation in the response to EGR1 was observed for construct 255pCAT6, compared to the construct 892pCAT6 (Figure 4C). Nevertheless, these functional overexpression analyses point out that binding sites within minimal promoter region are sufficient for mediating *SOX18 trans*-activation by EGR1 in both model systems used in this study.

We have previously shown that Sp3 transcription factor, an another zinc-finger binding protein, is able to moderately down-regulate *SOX18* promoter activity in HeLa cells. Since both Sp3 and EGR1 recognize cluster of overlapping binding sites covered by EGR1 IV probe (-29 to +10 relative to *tsp*) (Figure 3A), we have tested whether EGR1 overexpression could overcome Sp3 inhibitory effect on *SOX18* promoter activity. Accordingly, we have performed functional competition assays in which both HeLa and EA.hy926 cells were co-transfected with fixed amount of Sp3 expression vector and increasing amounts of EGR1 expression vector. As presented in Figure 4D, overexpression of EGR1 causes dose dependent re-

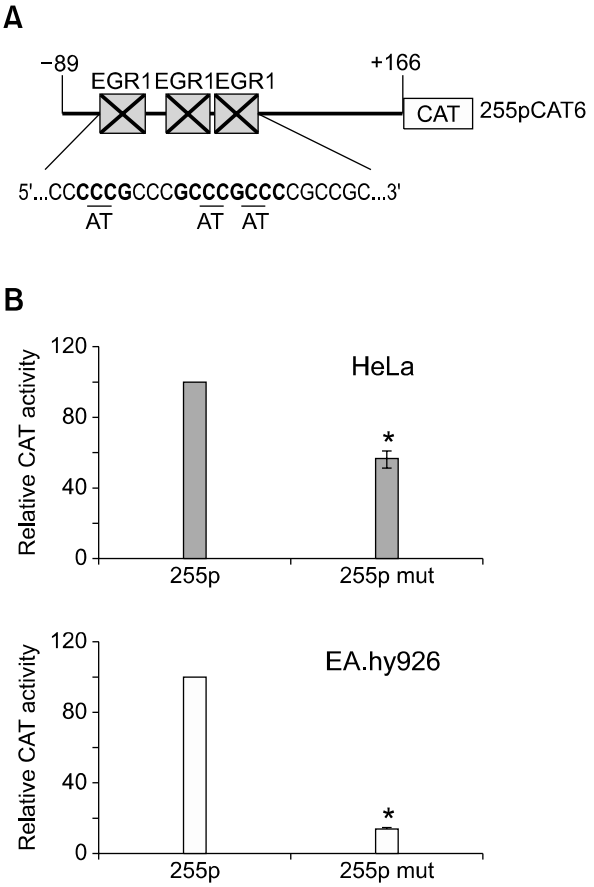

Figure 5. The effect of site directed mutations in EGR1 binding sites. (A) Schematic representation of the mutant construct 255mutCAT6 used in this study. (B) Transient transfection of HeLa and EA.hy926 cells with either *wild type* 255pCAT6 construct or its mutated counterpart 255mutCAT6. Normalized CAT activities were calculated as percentages of the *wild type* 255pCAT6 construct activity, which was set as 100%. Relative CAT activities are presented as the means ± SEM of three independent experiments. Values of $P < 0.001$ are presented by*.

activation of *SOX18* promoter up to 5.9- and 2.5-fold in HeLa and EA.hy926 cells, respectively. Our results demonstrate that overexpression of EGR1 transcription factor is capable to overcome Sp3-mediated repression, providing further evidence that EGR1 plays a critical role in *trans*-activation of the *SOX18* promoter activity.

Functional analysis of clustered EGR1 binding sites within the human *SOX18* minimal promoter region

In order to further determine the functional role of EGR1 in the regulation of *SOX18* expression, we introduced site directed mutations into cluster of three overlapping EGR1 binding sites within construct 255pCAT6 (Figure 5A). The ability of the mutant reporter construct and its wild-type coun-

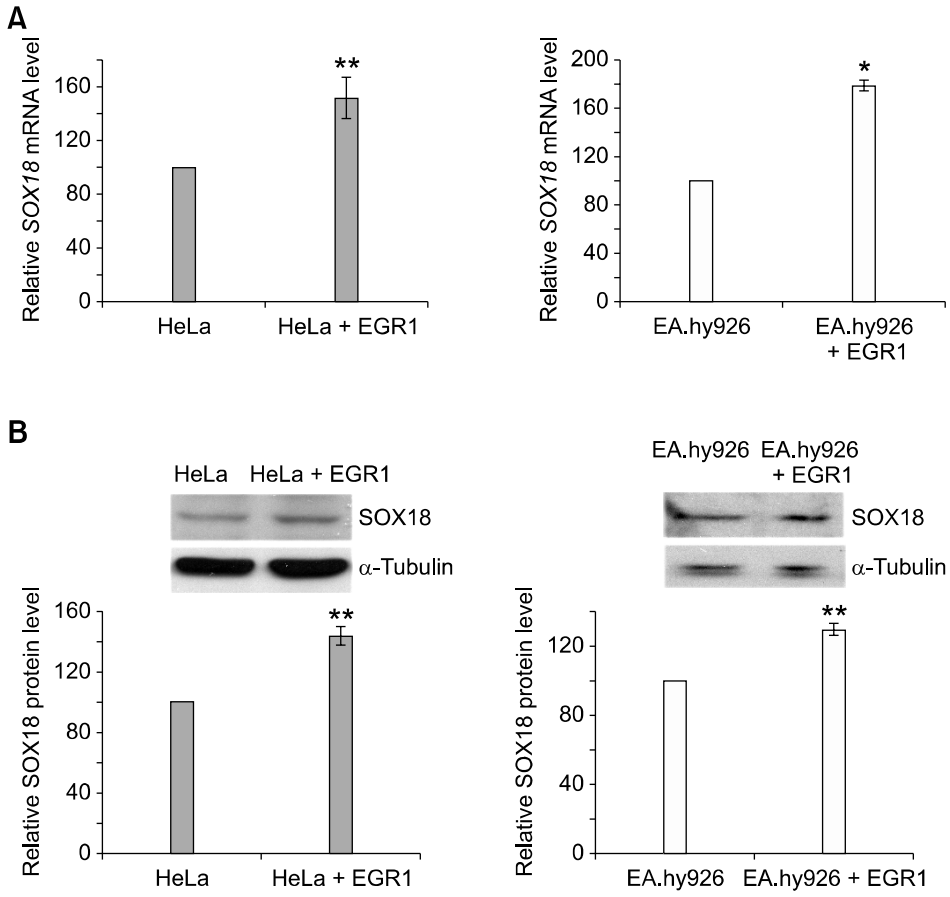

Figure 6. The effect of EGR1 on SOX18 expression in native context. (A) qRT-PCR analysis of *SOX18* transcription after transfection of HeLa and EA.hy926 cells with either empty pcDNA3.1 or pcDNA3.1-EGR1 expression vectors. The quantities of *SOX18* transcript in cells overexpressing EGR1 were calculated as a percentage of mock transfected cells which was set as 100%. Data of three independent experiments are presented at histograms as the means ± SEM. Values of $^{**}P < 0.05$ are presented by and values of $^{*}P < 0.001$ are presented by. (B) Western blot analysis of SOX18 expression after transfection of HeLa and EA.hy926 cells with either empty pcDNA3.1 or pcDNA3.1-EGR1 expression vectors. Detected proteins are indicated by arrows. The quantities of SOX18 protein in cells overexpressing EGR1 were calculated as a percentage of mock transfected cells which was set as 100%. Data of three (HeLa) or two (EA.hy926) independent experiments are presented at histograms as the means ± SEM. Values of $P < 0.05$ are presented by**.

terpart to drive the expression of reporter gene was analyzed in both HeLa and EA.hy926 cell lines. Mutations in EGR1 binding sites reduced promoter activity to 56% and 14% in HeLa and EA.hy926 cell lines, respectively (Figure 5B). These results indicate that EGR1 binding sites clustered immediately upstream of *tsp* are of functional relevance for the activity of *SOX18* minimal promoter region, while the remarkable effect observed in EA.hy926 cells could indicate the vital importance of this control element in the regulation of *SOX18* gene expression in endothelial system.

EGR1 overexpression increases *SOX18* mRNA and protein levels

We have performed real time RT-PCR and western blot analysis in order to determine the effect of EGR1 overexpression on *SOX18* mRNA and protein levels in HeLa and EA.hy926 cells. Quantitative real time RT-PCR has demonstrated that EGR1 overexpression leads to increase in *SOX18* mRNA level and leads to induction of *SOX18* gene transcription by approximately 1.5- and 1.8-fold in HeLa and EA.hy926 cells, respectively (Figure 6A). Importantly, 1.4- and 1.3-fold increase in *SOX18* protein level was also detected in HeLa and EA.hy926 cells, respectively (Figure 6B). These results signify the functional importance of EGR1 in activation of *SOX18* gene expression in a native context.

Discussion

Transcription factors serve as master switches for regulating a number of developmental processes (Oettgen, 2001). Along with others, transcription factor *SOX18* is shown to be involved in regulation of vascular development and postnatal neovascularization (Downes and Koopman, 2001). It has been demonstrated that *SOX18* participates in the VEGF-Flk1 pathway of endothelial cells activation (Darby *et al.,* 2001). Although three *SOX18* target genes have been identified so far (Im *et al.,* 2001; Hosking *et al.,* 2004; Fontijn *et al.,* 2008) and the role of *SOX18* in angiogenesis is established (Young *et al.,* 2006), little is known regarding its

transcriptional regulation. Accordingly, the aim of this study was to carry out comprehensive functional characterization of the human *SOX18* promoter, including determination of transcription start point and identification of control elements involved in the regulation of *SOX18* gene expression, with an emphasis on angiogenesis-related transcription factors.

Our *in silico* analysis of putative transcription factor binding sites within *SOX18* promoter region, among other transcription factors, revealed six putative EGR1 binding sites. By *in vitro* binding assays and functional analyses we have shown that three putative binding sites, located in a cluster close to *tsp*, are functionally relevant and sufficient for EGR1-induced *SOX18* transcription. It is common for GC-rich promoter regions of genes such as *SOX18* to possess several binding sites for transcription factors belonging to EGR family that often overlap with the ones for other zinc-finger transcription factors such as specificity protein 1 (Sp1) and Wilms tumor 1 (WT1) (Silverman and Collins, 1999). *In vitro* studies suggest that these transcription factors can displace one another from many promoters depending on equilibrium determined by their concentration and binding affinity (Silverman and Collins, 1999).

Previously, we have shown that zinc-finger transcription factors Sp3 and ZBP-89 are capable of binding to overlapping sites within *SOX18* promoter region (Petrovic *et al.,* 2009). We have shown that these proteins act as down-regulators of *SOX18* promoter activity (Petrovic *et al.,* 2009). Here, we have presented up-regulation of *SOX18* promoter by EGR1, acting through the same overlapping region. EGR1 consensus sequence often overlap with Sp1/Sp3 binding sites in many EGR1 target genes (Akuzawa *et al.,* 2000). It has been reported that EGR1 binds to an element overlapping the Sp1 site in the PDGF-B promoter (Khachigian *et al.,* 1996). In these studies, Sp1 contributes to basal level of gene expression, whereas EGR1 functions as an inducible transcription factor, activated in response to vascular injury, displacing pre-bound Sp1 (Khachigian *et al.,* 1996). In this report we have demonstrated that EGR1 overexpression overcomes Sp3-mediated repression of *SOX18* promoter activity. Presented results indicate that competition between members of Sp and EGR protein families might play an important role in the regulation of *SOX18* gene expression. We would like to hypothesize that *SOX18* up- or down-regulation depends on the concentration of these proteins in the nucleus, probably governed by different stimuli. It was previously shown that EGR1 expression level is low in quiescent endothelial cells, but is dramatically increased upon exposure to different stimuli such as hypoxia, tissue injury and mechanical stress (Akai *et al.,* 1994; Khachigian *et al.,* 1996; Yan *et al.,* 1999). Similarly, *Sox18* expression is up-regulated after injurious stimuli in endothelial cells during wound healing and tissue repair (Darby *et al.,* 2001). Taken together, it is likely that in quiescent endothelium, *SOX18* basal transcription is governed by other GC-binding transcription factors, like Sp-family members, while upon stimulation, EGR1 acts as *trans*-activator of *SOX18* transcription. It is important to point out that the same mechanism has been previously reported and described in vascular systems (Silverman and Collins, 1999).

Here we present that EGR1 overexpression leads to the up-regulation of *SOX18* promoter activity in both tumor (HeLa) and endothelial (EA.hy926) cell line. However, we have observed the difference in the response of minimal promoter region construct between two cell lines. While 5' deletion of full length promoter up to -89 (construct 255pCAT6) did not cause any significant change in the response to overexpressed EGR1 in HeLa cells, this deletion led to decreased response in EA.hy926 cell line (Figures 4B and 4C). Nevertheless, mutations of clustered EGR1 binding sites within *SOX18* minimal promoter region remarkably impair promoter activity, particularly in EA.hy926 cells (Figure 5B), signifying the major importance of this regulatory region in EGR1-mediated activation of *SOX18* gene transcription in endothelial setting.

For both *SOX18* and EGR1 it has been shown to play important roles in angiogenesis and tumor growth (Lucerna *et al.,* 2006; Young *et al.,* 2006). Significant expression of these transcription factors has been found during wound healing (Khachigian *et al.,* 1996; Darby *et al.,* 2001) and in atherosclerotic lesions (McCaffrey *et al.,* 2000). By data presented in this study, we have established *SOX18* as a novel target gene regulated by EGR1 transcription factor. Besides *SOX18* promoter reported here, EGR1 binding elements have been identified in the promoters of several other genes involved in angiogenic processes including growth factors (fibroblast growth factor-2 and TGF-β), adhesion molecules (intercellular adhesion molecule 1) and cytokines (TNFα) (Kim *et al.,* 1989; Biesiada *et al.,* 1996).

Angiogenesis induced by factors produced in cancer cells has been recognized as a critical step in tumor growth, progression and methastasis (Carmeliet, 2005). Since recent research demonstrated that *SOX18* is expressed during initial steps

of tumor vascularization, this gene has already been recognized as potential target for anti-angiogenic therapy (Young *et al.,* 2006). In parallel, other studies provided evidence for important role of EGR1 in microvascular endothelial cell growth, neovascularization, tumor angiogenesis and tumor growth (Fahmy *et al.,* 2003), identifying this protein as an another potential therapeutic target. In this study, for the first time, we have provided a functional link between *SOX18* and EGR1, two potential targets in anti-angiogenic therapy. Thus, clarifying the complex mechanisms involved in transcriptional regulation of human *SOX18* gene could help in better understanding of important physiological and patho-physiological processes in which *SOX18* gene participates.

Taken together, we have determined unique *SOX18* transcription start point, defined minimal promoter region indispensable for *SOX18* transcription and revealed the presence of essential regulatory element(s) positioned between -89 and +29 relative to *tsp*. Furthermore, by functional analyses and site directed mutagenesis we have demonstrated the important role of EGR1 in the up-regulation of *SOX18* promoter activity. Finally, we have shown that EGR1 up-regulates *SOX18* expression in a native context on both mRNA and protein level. We believe that presented results provide an important contribution in understanding of *SOX18* gene transcriptional regulation, since this gene is marked as a potential anti-angiogenic target.

Methods

Cell culture

HeLa (ATCC CCL 2) were maintained in DMEM supplemented with 10% FBS and 1 × MEM nonessential amino acids at 37°C in 5% CO_2. EA.hy926 cells (Edgell *et al.,* 1983) were maintained in DMEM, supplemented with 10% FBS and hypoxanthine/aminopterin/thymidine (HAT supplement) at 37°C in 5% CO_2.

Primer extension analysis

Antisense primers used in primer extension reactions were: PE5: 5'-TCGTCCTGTGCTCCGTAGCCGGGCGGC--3' (+35), PE3: 5'-AAGGGCAGGCCAGGCCGGGAGGGCGGATG-3' (-78).

The numbers indicated in parenthesis correspond to the distance in nucleotides from 5' end of the sequence to the A of the first ATG. Primers were end labeled with [γ^{32}P] ATP. 20 μg of total cellular RNA prepared either from EA.hy926 or HeLa cells (Rnase mini Kit, QIAGEN) was mixed with radiolabeled primer (10^5 cpm) and incubated at 65°C for 5 min. The annealing mix was cooled on ice, and primers were extended with 200 units of SuperScript™ RnaseH-reverse transcriptase (Invitrogen) for 1 h at 37°C in a reaction containing 30 units of RNAguard™ (Amersham Pharmacia Biotech), 2 mM dNTP mix, 10 mM DTT, 50 mM Tris-HCl (pH 8.3), 75 mM KCl and 3 mM $MgCl_2$. Control reaction was performed with 10 μg of yeast tRNA. The lengths of the primer extension products were determined by comparison to dideoxy-sequencing reaction performed using the same primers (T7 Sequencing Kit, USB) as well as to the radiolabeled *PhiX/Hin*fIII DNA ladder.

Generation of *SOX18* promoter constructs

SOX18 promoter fragments *Pst*I-*Pvu*II (892 bp), *Pst*I-*Stu*I (637 bp), *Stu*I-*Pvu*II (255 bp) and *Nar*I-*Pvu*II (137 bp) were first cloned in pBSKS+ vector. Fragments were subsequently released by *Hind*III-*BamH*I, except for the fragment *Nar*I-*Pvu*II that was released by *Sa*II-*BamH*I, and cloned in pBLCAT6 to generate clones 892pCAT6, 637pCAT6, 255pCAT6, and 137pCAT6. Mutated fragment 255mut was commercially synthesized (GeneScript Corporation), cloned into pUC57 vector and subsequently subcloned into pBLCAT6 vector in *Hin*dIII/*Bam*HI sites.

In silico analysis of the *SOX18* 5' upstream regulatory region

MatInspector Release professional 7.2.2 (http://www.genomatix.de/) was used to analyze putative transcription factor binding sites within the *SOX18* promoter region.

Transient transfection assays

HeLa cells were transfected using calcium-phosphate precipitation method. 1.2×10^6 cells were seeded in 10 cm dish (two dishes per transfection) and transfected with 10 μg of various *SOX18* promoter constructs, together with 3 μg of pCH110 vector (Amersham Pharmacia Biotech) and 4 μg of pBluescript (Stratagene). In co-transfection assays 2 μg of either empty pcDNA3.1 or pcDNA3.1-EGR1 expression vector was used. EA.hy926 cells were transfected using LIPOFECTAMINE reagent (Invitrogen). For each transfection, 2×10^6 cells were seeded into 6-cm dish and 4.5 μg of various *SOX18* promoter constructs together with 1.5 μg of pCH110 vector were mixed with 16 μl of LIPOFECTAMINE reagent. In co-transfection assays, 0.8 μg of either empty pcDNA3.1 or pcDNA3.1-EGR1 expression vector was used. In functional competition assays, both HeLa and EA.hy926 cells were co-transfected with fixed amount of Sp3 expression vector (4 μg and 0.8 μg, respectively) and increasing amounts of EGR1 expression vector, where Sp3:EGR1 ratios were 1:0.5 and 1:1. Extracts for β galactosidase and chloramphenicol acetyltransferase (CAT) assays were prepared after 24 h and 48 h for EA.hy926 and HeLa cells, respectively. β galactosidase and CAT assays were performed as described (Kovacevic Grujicic *et al.,* 2005). The normalized CAT activities were evaluated as a percentage of the selected promoter construct which was set as 100% activity. Mean values of relative CAT activities were compared with Student's *t* test using SPSS software. A

P-value of less than 0.05 was considered significant.

Nuclear extract preparation and electrophoretic mobility shift assays (EMSA)

Nuclear extracts from HeLa cells were prepared according to standard procedure (Dignam *et al.,* 1983). The following sense oligonucleotides were used in the EMSA and supershift studies: EGR probe I: 5'-CAAGGGCCCTTGGGGGGCAGGGAGGACG 3' (-665), EGR probeII: 5'-GAGCCTCCCAGCGGGGGGCGGGGAACGGCAA 3' (-303), EGR probe III: 5'-GGGGGAGGTGGGGGGGCTGTGCGCGGGGGAGG 3' (-121), EGR probe IV: 5'-GACCCGCCCCCGCCCGCCCGCCCCGCCGCCGATTGG 3' (-29), Consensus EGR1: 5'-GGATCCAGCGGGGGCGAGCGGGGGCGA 3'.

The numbers indicated in parenthesis correspond to the distance in nucleotides from 5' end of the sequence to the transcription start point (*tsp*). All antisense oligonucleotides were designed to contain 2∼3 unpaired G nucleotides at 5' end and probes were labeled by fill-in reaction. EMSA experiments were performed as described (Kovacevic Grujicic *et al.,* 2005). For supershift assay, EMSA reaction was preincubated for 30 min at 37°C with 2 μg of anti-EGR1 antibody (Cell Signaling Technology, #4154).

Western blot analysis

Whole cell lysates (WCL) were prepared after transient transfection of HeLa and EA.hy926 cells with either empty pcDNA3.1 or pcDNA3.1-EGR1 expression vectors. Cells were collected and lysed with lysis buffer containing 50 mM Tris-Cl pH 8.0, 150 mM NaCl, 1% NP-40, 5 μg/ml PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 μg/ml pepstatin. Proteins were separated on 10% SDS-PAGE. Membranes were blocked in 10% non-fat milk for 1 h, following the incubation with anti-SOX18 antibody (sc-20100, Santa Cruz Biotechnology) for 1 h at room temperature. Mouse anti-α-tubulin Ab (CP06 - Calbiochem) was used for normalization of protein amount. Immunoreactive bands were detected using HRP-conjugated anti-rabbit IgG (Amersham Pharmacia Biotech) or anti-mouse IgG (Amersham Pharmacia Biotech) and ECL detection kit (Amersham Pharmacia Biotech). Immunoblots were digitalized and quantified with ImageQuant Version 5.2 software and normalized for α-tubulin values. The quantities of SOX18 protein were calculated as a percentage of mock transfected cells which was set as 100%. Mean values of relative SOX18 expression were compared with Student's *t*-test using SPSS software. A *P*-value of less than 0.05 was considered significant.

Two-step qRT-PCR

Total RNAs from HeLa and EA.hy926 cells, either mock transfected or transfected with EGR1 expression construct, were isolated using TRI-Reagent (Ambion) and 1 μg of each RNA was reverse transcribed using MuLV reverse transcriptase (Applied Biosystems) and random hexamers (Applied Biosystems). cDNAs were subjected to real time PCR using Power SYBR Green PCR Master Mix (Applied Biosystems) in 7500 Real-Time PCR Systems (Applied Biosystems). *SOX18* cDNA was amplified using following primers: forward, 5' TTCCATGTCACAGCCCCCTAG 3', and reverse 5' GACACGTGGGAACTCCAG 3'. GAPDH used as endogenous control was amplified using following primers: forward, 5' GGACCTGACCTGCCGTCTAG 3' and reverse, 5' CCACCACCCTGTTGCTGTAG 3'. All samples were measured in triplicates and the mean value was considered. The relative level of SOX18 expression was determined using comparative quantification algorithm where resulting ΔΔCt value was incorporated to determine the fold difference in expression ($2^{-\Delta\Delta Ct}$). Relative SOX18 expression was presented as percentage of SOX18 expression in mock transfected cells that was set as 100%. Values were compared with Student's *t* test using SPSS software and *P*-value of less than 0.05 was considered significant.

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