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Sp1 mediate hypoxia induced ephrinB2 expression via a hypoxia-inducible factor independent mechanism

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ABSTRACT

Environmental factors are instrumental in maintaining a healthy vasculature. Oxygen tension is higher in arteries than in veins and thus has the potential to be an instructive signal in arterial/venous specification. EphrinB2 is specifically expressed in arteries and required during embryonic vessel formation. In this study, we show that expression of ephrinB2 is oxygen dependent. Mutagenesis of hypoxia-responsive elements and transactivation experiments determined this regulation to be achieved in a hypoxia-inducible factor independent manner. MAZ and Sp1 are known to regulate transcription together and have been shown to bind to the same sites within promoters. Chromatin immunoprecipitation confirmed that binding of Sp1 to the ephrinB2 promoter was favored compared to MAZ under hypoxic relative to normoxic conditions. Furthermore, siRNA mediated knockdown of Sp1 attenuated this hypoxic response. These results indicate that hypoxia drives arterial differentiation by increasing ephrinB2 expression in endothelial cells through Sp1 activation.

Introduction

The endothelial precursor cells forming the primitive vasculature display arterial/venous (AV) specificity before the onset of blood flow, indicating that there is a genetic component to AV determination. However, several studies suggest that AV identity of endothelial cells is not only intrinsic but rather is plastic and depends on environmental cues within the vascular network [1,2]. Oxygen tension, as well as hemodynamic forces, is micro environmental determinants that could be implicated in these processes.

EphrinB2 is differentially expressed in arteries whereas veins preferentially express the cognate receptor EphB4. This reciprocal Ephrin/Eph signaling is crucial in vascular development as deleting either of the genes results in lethality by embryonic day 11 due to defects in blood vessel remodeling [3–5]. We have recently characterized the regulatory sequences and transcription factors required for the expression of ephrinB2 during basal transcription [6]. Two of these, Myc-associated zinc finger protein (MAZ) and Stimulating protein 1 (Sp1), are known to regulate transcription together and have been shown to bind to the same sites within promoters [7–9]. Furthermore, it has recently been published that shear stress in endothelial progenitor cells induced ephrinB2 expression through Sp1 [10].

In the present study, we investigate the mechanisms that control the hypoxic regulation of ephrinB2 gene expression. We found this regulation to be independent of hypoxia-inducible factor but could show the hypoxic effect to be reliant on Sp1 replacing MAZ on the promoter.

Materials and methods

Cell culture under normoxic and hypoxic conditions. Mouse arterial endothelial cells (MAE cells) were grown in medium (Gibco, 31966) with 10% fetal calf serum at 37 °C in humidified atmosphere with 5% CO_2. Confluent cells were incubated in 12-well plates under normoxia, 1%, 3% or 5% oxygen for 24 h. The experiments were made in triplicates. RNA was extracted from the cells using the RNeasy Micro Kit (Qiagen) and the RNA was used to synthesize cDNA using the Superscript III First-Strand kit (Invitrogen). The cDNAs were then used for qPCR analysis.

Mutation construct. A putative hypoxia-responsive element (HRE) was found 978 bp upstream of the transcription start of the ephrinB2 gene. This site was mutated in a pGL3 plasmid containing 2.3 kb of ephrinB2 upstream sequence fused to the luciferase gene. The mutation was introduced using the Quickchange II site-directed mutagenesis kit (Stratagene), according to the manufacturer's

Abbreviations: EC, endothelial cell; MAE cells, mouse arterial endothelial cells; Chip, chromatin immunoprecipitation; PCR, polymerase chain reaction; HIF, hypoxia-inducible factor ; Sp1, stimulating protein 1; MAZ, MYC-associated zink finger protein

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instructions. The primers used were; CAGCATTGTAAGAAGAGAAA AGGTTAATTTTTGCTG and GCCCATGGCCCAGCAAAAATTAACCTTT TCTCTTCT.

Plasmid DNA was prepared using the Qiaprep Spin Miniprep kit (Qiagen), and clones were sent to MWG biotech AG for sequencing.

Luciferase assay. MAE cells were grown in 12-well plates, at ~80% confluency they were transfected using the profection mammalian transfection system—calcium phosphate (Promega) according to the manufacturers instructions. The experiment was made in triplicates and 1 μg of plasmid DNA was used for each transfection. The luciferase constructs were cotransfected with 0.5 μg of a plasmid containing the lacZ gene under control of the β-actin promoter. After 24 h the transfection medium was replaced by fresh medium. One plate containing cells that had been transfected with the wt construct or the HRE mutation construct was incubated in 1% oxygen for 24 h, while another identical plate was incubated under normoxic conditions. In addition, MAZ and Sp1 mutation constructs were tested under hypoxic conditions. These constructs have been described previously [6]. Luciferase assays were performed using the dual-light system (Applied Biosystems) according to the manufacturer's instructions. The β-gal and luciferase activity was measured using an anthos lucy 1 microplate luminometer (Anthos labtec instruments). The β-gal values were used to normalize the luciferase values.

Transfection of HIF-constructs. Plasmids expressing genes for HIF-1α or HIF-2α were transfected into MAE cells at ~80% confluency using the profection mammalian transfection system—calcium phosphate (Promega) according to the manufacturer's instructions. Untransfected and mock-transfected cells were also used. The HIF-constructs contain genes that have been modified so that the gene products are not degraded in normoxia. For the mock transfection a GFP-expressing plasmid was used in order to determine the transfection efficiency. The experiment was made in triplicates and 1 μg of plasmid DNA was used for each transfection, transfection efficiency was ~50%. Cells were harvested after 20 h and RNA was extracted from the cells using the RNeasy Micro Kit (Qiagen), the RNA was used to synthesize cDNA using the Superscript III First-Strand kit (Invitrogen). The cDNAs were then used for qPCR analysis.

Chromatin immunoprecipitation assay. Chip assays were performed for the transcription factors Sp1 and MAZ. The assays were done using the EZ-Chip kit (Upstate) according to the manufacturer's instructions. Confluent MAE cells (90%) were incubated in a humidified chamber in normoxia or 1% oxygen and 5% CO_2 for 24 h, then 2×10^6 MAE cells were lysed in 1 ml of lysis buffer and sonication was done in 1.5 ml microfuge tubes, 250 μl in each tube. The sonicator used was a bioruptor (Diagenode), and the settings were: high, 30 s on, 30 s off for 7.5 min. The program was run twice. The resulting DNA lengths were ~200–1000 bp, and 100 μl of sonicated DNA was used for each assay. The anti-bodies used were: MAZ (Santa Cruz Biotechnology sc-28745) and Sp1 (Upstate, 07-645). The DNA retrieved was subsequently used for qPCR analysis.

Western blot. The siRNA transfected and untreated MAE cells were lysed in 100 μl NETN buffer (0.5% Nonidet P-40/1 mM EDTA/20 mM Tris–HCl, pH 8.0/100 mM NaCl) and protein concentration was measured using Bio-rad protein assay reagent and a Thermomax microplate reader (Molecular devices). Protein levels were adjusted for equal loading. Proteins were resolved on 10% SDS–PAGE gels and transferred to nitrocellulose membranes. Primary anti-bodies used were MAZ (Santa Cruz Biotechnology, sc-28745) and Sp1 (Upstate, 07-645). For detection secondary horseradish peroxidase coupled anti-bodies were used.

SiRNA. siRNA against Sp1, MAZ (Dharmacon, L-040633-01, L-059837-01) and a negative control siRNA (against ECFP) was transfected into MAE cells using Dharmafect1 transfection reagent (Dharmacon, T-2001-02) according to the manufacturers instructions. A 12-well plate was used, the experiment was done in triplicates and the cells were grown in transfection medium for 48 h. Then fresh medium was added to the cells and they were incubated at 1% oxygen for 24 h. RNA was extracted from the cells using the RNeasy Micro Kit (Qiagen) and the RNA was used to synthesize cDNA using the Superscript III First-Strand kit (Invitrogen). The cDNAs were then used for qPCR analysis.

Quantitative RT-PCR. qPCR was performed using Applied Biosystem 7000. Sybr green PCR master mix (Applied Biosystems) was used for all qPCRs in 25 μl reactions. DNA (2 μl) from each of the chip assays were loaded, the primers used was: AGTGGCTAA GAGGCGACCA and CGCGCTGTCAGAGCACTAT. DNA from these reactions was also resolved on a 1.5% agarose gel. For the siRNA and HIF-transfection experiments equal amounts of cDNA were loaded and normalized to β-actin. The primers used were ephrinB2: AGGAATCACGGTCCAACAAG and ACTTCGGAACCCAGGAG ATT, β-actin: TGTTACCAACTGGGACGACA and GGGGTGTTGAAGG TCTCAAA,

Statistics. The experiments presented in Figs. 2, 3, and 4B are all triplicate experiments. The error-bars in these figures represent standard error of the mean, SEM.

Results

Induction of ephrinB2 expression in hypoxia

We have previously studied the ephrinB2 promoter during normoxic conditions and found several transcriptionally active elements in the promoter [6]. Since it has been shown that EphrinB2 is up-regulated during hypoxic conditions in other cells [11], we searched the upstream sequence of ephrinB2 for putative hypoxia-responsive elements (HREs). One putative HRE was found 978 bp upstream of the transcription start site. A schematic representation of the ephrinB2 promoter is shown in Fig. 1A. In addition, incubation of MAE cells in normoxia, 1%, 3%, and 5% oxygen showed that ephrinB2 is equally strongly up-regulated in different levels of hypoxia (Fig. 1B).

HIF-1α and HIF-2α does not induce ephrinB2 expression in MAE cells

The putative HRE found in the ephrinB2 upstream sequence was mutated in a luciferase construct. The mutation construct and wt construct were transfected into MAE cells, which subsequently were incubated in normoxia or hypoxia. We could not detect any difference in luciferase activity between the wt and mutation constructs in normoxia or hypoxia (Supplementary data). In order to further investigate weather HIF-1 or HIF-2 is involved in ephrinB2 induction, plasmids expressing HIF-1α or HIF-2α were transfected into MAE cells. The genes expressed by these vectors are modified versions, so the proteins are not degraded in normoxia. Transfections of neither the Hif-1α nor Hif-2α construct caused any induction of ephrinB2 expression (Supplementary data).

Identification of regulatory elements in the ephrinB2 promoter during hypoxia

We have previously shown that MAZ is required for basal ephrinB2 promoter activity and that Sp1 does not bind to the ephrinB2 promoter in MAE cells under normoxic conditions even though the promoter contains two active Sp1 binding sites [6]. However, as Sp1 is known to be involved in inducing gene expression in response to hypoxia [12,13] and MAZ also can regulate promoter activity by binding to Sp1 sites [9], we wanted to address whether

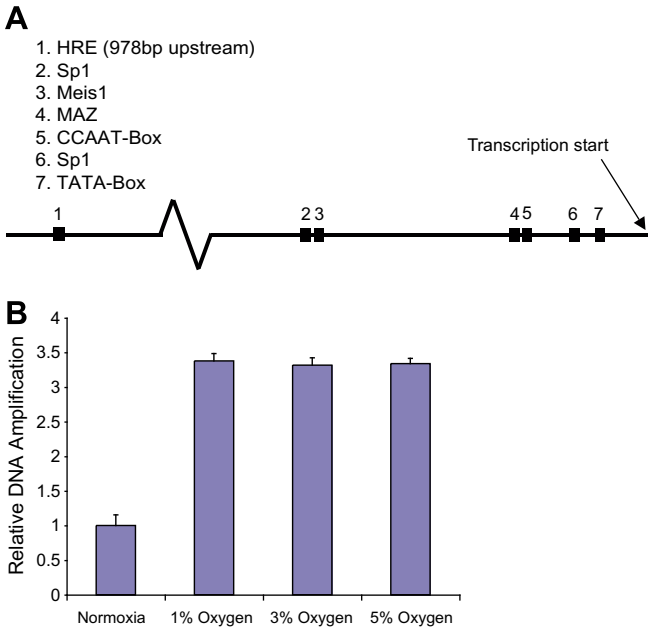

Fig. 1. (A) Schematic representation of the ephrinB2 promoter. Box 1 represents a putative hypoxia-responsive element located 978 bp upstream of the transcription start site. Boxes 2–7 represent transcriptionally active sites that we have described previously [6], and they all lie within 180 bp upstream of the transcription start site. (B) In order to investigate the hypoxic regulation of ephrinB2 in MAE cells, we incubated MAE cells for 24 h in normoxia, 1%, 3%, and 5% oxygen. Subsequent qPCR on cDNA from these cells showed that ephrinB2 expression is equally upregulated in response to the different levels of hypoxia. The putative HRE was mutated in a luciferase construct that was tested in MAE cells, but the element was shown to not be transcriptionally active in MAE cells (Supplementary data). Also, plasmids expressing Hif-1α or Hif-2α were transfected into MAE cells. This experiment showed that neither HIF-1α nor HIF-2α induced expression of ephrinB2 (Supplementary data).

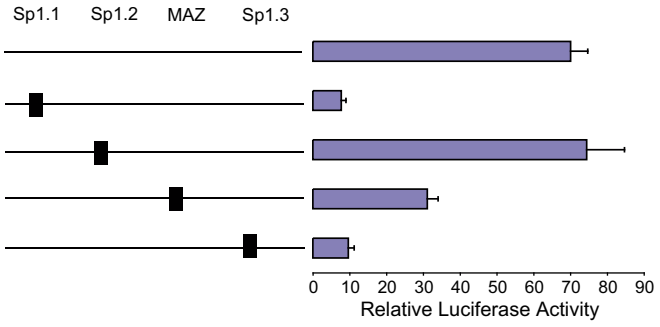

Fig. 2. We have previously used mutation constructs to identify several elements in the ephrinB2 promoter that are transcriptionally active in normoxia [6]. These mutation constructs were used to reveal MAZ and Sp1 sites that are active during hypoxic conditions. We could show that the same sites that are active in normoxia are also active during hypoxia.

these sites were transcriptionally active in the ephrinB2 promoter during hypoxia. Using mutation constructs targeting Sp1 and MAZ elements we could show that these sites are active during hypoxia (Fig. 2).

Sp1 binds to the ephrinB2 promoter in hypoxia

Since MAZ can regulate promoter activity by binding to Sp1 sites [9], we hypothesized that the observed MAZ binding during normoxia involves Sp1 sites in the ephrinB2 promoter and that during hypoxia MAZ is replaced by Sp1 on the promoter, thus

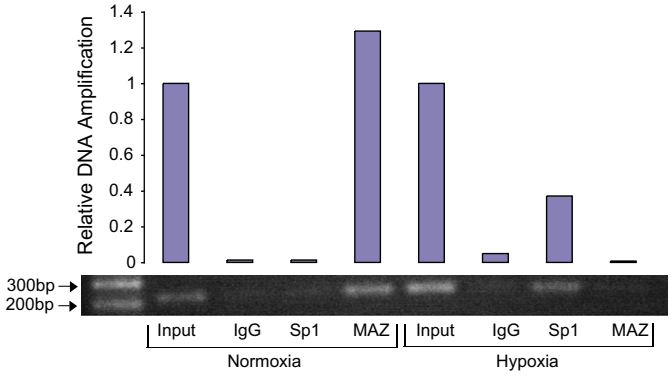

Fig. 3. Chromatin immunoprecipitation experiments were performed on MAE cells that had been incubated in normoxia or 1% oxygen for 24 h. Anti-bodies against Sp1 and MAZ were used and qPCR analysis was performed on the DNA retrieved from the experiments. This analysis showed that MAZ but not Sp1 binds to the promoter during normoxia while Sp1 but not MAZ interacts with the promoter in during hypoxic conditions. Running DNA from the qPCR reactions on a 1.5% agarose gel also proved the amplified DNA fragments to have the expected size (223 bp). This figure shows a representative experiment out of 3 made.

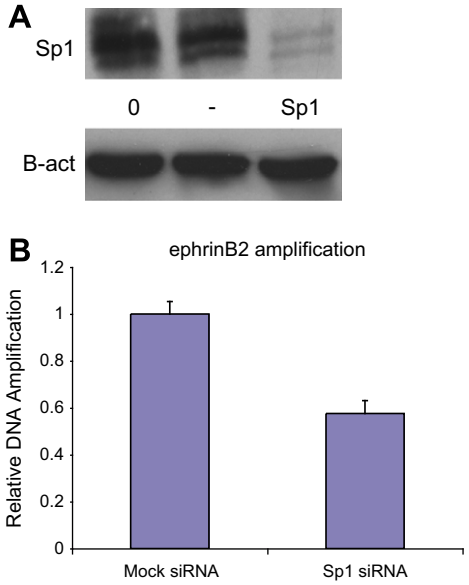

Fig. 4. MAE cells were transfected with siRNA against Sp1 while incubated in normoxia for 48 h. Then the cells were incubated in 1% oxygen for 24 h before they were harvested. (A) A Western blot indicates significant down regulation of Sp at the protein level. (B) Quantification of EphrinB2 transcripts using qPCR revealed a 42% reduction in response to Sp1 knockdown.

inducing ephrinB2 expression. In order to test this hypothesis, ChIP (chromatin immunoprecipitation) was performed on normoxic and hypoxic MAE cells using anti-bodies against Sp1 and MAZ. These experiments show that MAZ but not Sp1 binds to the promoter during normoxia while Sp1 but not MAZ binds to the promoter during hypoxia (Fig. 3).

Knocking down Sp1 in hypoxic MAE cells reduces ephrinB2 expression

To validate our ChIP results siRNA experiments were designed to test the role of Sp1 in hypoxia with regard to ephrinB2 expression. siRNA against Sp1 were transfected into MAE cells which subsequently were incubated under hypoxic conditions. Down regulation of Sp1 was conformed at both mRNA and protein level in response to siRNA treatment (Fig. 4A). Quantification of

ephrinB2 transcripts using qPCR proved that ephrinB2 expression was indeed down regulated in response to knock down of Sp1 during hypoxia (Fig. 4B).

Discussion

The zinc finger proteins Sp1 and MAZ are transcription factors that control the expression of various genes. Regulation of transcription by these factors is based on interactions between GC-rich DNA-binding sites and the carboxyl-terminal zinc finger motifs of the two proteins [8]. MAZ and Sp1 are known to regulate transcription together and they have been shown to bind to the same sites within promoters [7,9]. In addition, Sp1 has previously been shown to be involved in hypoxic regulation of other genes [12,13].

Hypoxia is an important factor for driving angiogenesis both during embryogenesis and in the adult [14,15]. Angiogenesis during embryonic development is a normal process, while angiogenesis in the adult often takes place in response to a pathologic process. Uncovering mechanisms for hypoxic induction of angiogenesis may not only be important for understanding embryonic vascular development, but also mechanisms behind pathological processes in the adult. In this study we aimed to investigate the mechanism for hypoxic induction of ephrinB2, a vital gene for correct vascular development [5]. By use of *in vitro* transfection, mutagenesis techniques, chromatin immunoprecipitation and siRNA knockdown we showed that: (1) neither HIF-1α nor HIF-2α are responsible for hypoxic induction of ephrinB2 in MAE cells; (2) Sp1, but not MAZ, bind to the eprinB2 promoter during hypoxia; (3) knocking down Sp1 reduces ephrinB2 expression in hypoxia.

During later developmental stages and in the adult, arteries exist in a high oxygen pressure environment. Several genes, including ephrinB2, that are induced in the arterial compartment during hypoxic conditions are still expressed in high oxygen conditions in adult arteries [16]. However, at this time point a functioning circulation has been established and a recent publication Obi et al. could show specific binding of Sp1 to the ephrinB2 promoter in response to shear stress inducing arterial differentiation of endothelial progenitor cells [10]. Thus, regulating ephrinB2 expression by Sp1 either induced by hypoxia or shear stress allows for the correct arterial induction during development as well as maintenance of the arterial phenotype in the adult.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.10.146.

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