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## SOX9 mediates the retinoic acid-induced HES-1 gene expression in human breast cancer cells

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**Abstract** We have previously shown that the anti-proliferative effect of retinoic acid in human breast cancer cell line MCF-7 is dependent on HES-1 expression. Here we show that retinoic acid induces HES-1 expression via upregulation of transcription factor SOX9. By expressing a dominant negative form of SOX9, disrupting endogenous SOX9 activity, the retinoic acid-induced HES-1 mRNA expression was inhibited. We found an enhancer regulating HES-1 expression: two SOX9 binding sites upstream of the HES-1 gene that were capable of binding SOX9 *in vitro*. By performing chromatin immunoprecipitation, we showed that SOX9 binding to the HES-1 enhancer was induced by retinoic acid *in vivo*. In reporter assays, transfection of a SOX9 expression plasmid increased the activity of the HES-1 enhancer. The enhancer responded to retinoic acid; furthermore, the expression of a dominant negative SOX9 abolished this response. Taken together, we present here a novel transcriptional mechanism in regulating hormone-dependent cancer cell proliferation.

**Keywords** atRA · HES-1 · SOX9 · Proliferation

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### Introduction

Retinoic acid belongs to a class of compounds of both synthetic and naturally occurring molecules, the retinoids. Retinoids are non-steroid hormones, metabolites of vitamin A with intracrine activity. During embryonic development, the retinoids play a crucial role in regulating a variety of important cellular functions [1]. In both normal and malignant cells, the retinoids regulate cell growth and differentiation. These properties make retinoids promising as anti-tumorigenic and anti-carcinogenic agents, although certain side effects have hampered their clinical use [2–4]. Despite these potential drawbacks, all-*trans* retinoic acid (atRA), a naturally occurring form of retinoids, is used as a standard treatment for acute promyelocytic leukemia.

All-*trans* retinoic acid exerts its biological effect, like other retinoids, through binding and subsequent activation of the retinoic acid receptors (RARs). Three RAR subtypes,  $\alpha$ ,  $\beta$ , and  $\gamma$ , have been shown to mediate cellular responses to retinoids. In the absence of a ligand, the RARs bind to specific DNA elements, retinoic acid response elements (RAREs), in the *cis*-regulatory region of target genes as heterodimers together with retinoid X receptors (RXRs) [5]. The heterodimers associate with the corepressors SMRT and N-CoR followed by a recruitment of histone deacetylases resulting in transcriptional repression [6]. When bound to ligand, RAR–RXR heterodimers recruit coactivators, followed by transcriptional activation of gene expression [7].

Many cancer cell lines respond to retinoic acid with growth arrest, induction of apoptosis, or differentiation [8]. Growth arrest is mainly accomplished by blocking cell cycle progression in the G1 phase [9]. It is well established that retinoic acid inhibits the proliferation of breast cancer cells [10]; however, the sensitivity to retinoic acid of

estrogen receptor  $\alpha$  (ER $\alpha$ )-positive cells contrasts to that of ER $\alpha$  negative cells, where the majority of the cell lines are resistant [11, 12]. ER $\alpha$ -positive cells are sensitive to retinoic acid since ER $\alpha$  expression correlates with RAR $\alpha$  expression [12], as shown by the fact that exogenous expression of ER $\alpha$  in ER $\alpha$ -negative cells induces RAR $\alpha$  expression and thus restores sensitivity to retinoic acid [13]. The retinoic acid-induced block of G1 to S phase transition has been associated with regulation of cell cycle modulators: increased expression of p21, hypophosphorylation of RB, decreased activity of cdk2 and cdk4, decreased expression of cyclin B1, D1, D3, and E2F-1 [14, 15].

We have previously shown that ectopic expression of the basic helix–loop–helix transcriptional repressor *Hairy* and *Enhancer of Split* homologue-1 (HES-1) inhibits estrogen-stimulated proliferation [16] and estrogen-induced expression of the cell cycle regulator E2F-1 [17]. Moreover, the anti-proliferative effect of retinoic acid is dependent on HES-1 in the breast cancer cell line MCF-7 [18]. In PC12 cells, HES-1 arrests cell growth as well as nerve growth factor-induced differentiation [19]. Increasing levels of HES-1 protein expression, accomplished by using doxycyclin-inducible HES-1 expression system, resulted in increased growth inhibition of human pulmonary carcinoid cancer cells [20]. As a primary Notch effector protein, HES-1 is well characterized as an inhibitor of neuronal differentiation [21] and HES-1 has also been shown to be essential in myogenesis [22, 23], eye morphogenesis [24], and T-cell lineage [25], in addition to its role as a regulator of proliferation.

The transcription factor SOX9, [SRY (sex determining region Y), box9], is essential in sex determination and during chondrogenesis. Mutations in the SOX9 gene causing disrupted expression result in autosomal XY sex reversal and in campomelic dysplasia, a syndrome with severely malformed skeleton [26]. SOX9 can substitute for SRY expression during sex determination, as shown by the ectopic expression of SOX9 in XX gonads of mice, which induced testis formation [27]. Effects on cell cycle progression by SOX9 have been reported in the prostate tumor cell line M12 [28] and in the chondrocytic cell line CFK2. The ectopic expression of SOX9 arrests cells in the G1 phase of the cell cycle and is associated with an upregulation of p21 gene expression [29]. Retinoic acid induces SOX9 expression in retinoid-sensitive breast cancer cell lines, and SOX9 has been shown to play a role in retinoid-induced growth inhibition in the human breast cancer cell line T47D. By inhibiting endogenous SOX9 with an ectopically expressed dominant negative SOX9, the anti-proliferative effect of retinoids was abolished. Overexpression of SOX9 in the same cell line, T47D, caused similar cell cycle changes as retinoid treatment [30].

To further characterize the molecular mechanisms of the anti-proliferative effects of retinoic acid, we examined the role of SOX9 in the regulation of HES-1 gene expression in human breast cancer cells. We found SOX9 to be a mediator of the atRA-induced HES-1 gene expression in MCF-7 cells. Furthermore, we found that SOX9 regulates HES-1 expression via a *cis*-regulatory element upstream of the HES-1 gene.

## Materials and methods

### Cell lines, transient transfections, and luciferase assays

MCF-7 and HEK 293 cells were maintained in Dulbecco's modified Eagle medium (GIBCO) supplemented with 10% fetal bovine serum (Saveen Warner AB) and 0.1% Gentamicin (GIBCO). Transient transfections were performed using Lipofectamine 2000 (Invitrogen) with  $4 \times 10^5$  seeded cells per 35 mm well and 100 ng of each plasmid DNA, in Opti-MEM (GIBCO). Total cell lysates were harvested 48 h later, and luciferase activity was measured by using Luciferase assay kit (Biothema) in a Berthold FB12 luminometer (Labsystem). The RSV- $\beta$ gal plasmid was included in each reaction as an internal control for transfection efficiency, and luciferase activity was normalized to  $\beta$ -galactosidase activity, which was measured by Power-Wave X micro plate reader (Bio-Tek Instruments Inc.). Each transfection experiment was done in triplicates and presented as mean values ( $\pm$ SD).

### Transfection of siRNA to SOX9

About 50,000 cells/well were seeded onto a 24-well plate, and on the subsequent day, the cells were transfected with 100 nM of siRNA targeting SOX9 (SOX9 smart pool) or GAPDH (5'-TGGTTTACATGTTCCAATA-3') by using DharmaFECT 2 transfection reagents according to the manufacturer's (Dharmacon) instruction. The next day, medium was changed, and after another 30 h, 1  $\mu$ M atRA was added for 6 h to the cells, as indicated in the figure. Expression of SOX9, HES-1, and GAPDH was quantified using real time PCR.

### Plasmids and chemicals

The 4 $\times$  SOX9RE and the 6 $\times$  SOX9RE contain four or six copies, respectively, of putative SOX9 response element (5'-AGGTCAACAAGGAGGCATTGTTTCATCA-3') inserted into pGL3 promoter vector (Promega). FLAG-tagged SOX9 and truncated SOX9 (tr.SOX9) were cloned into pcDNA3 as described [31]. mRAR $\alpha$  and mRXR $\alpha$  were inserted

into pSG5 vector. 17 $\beta$ -Estradiol (E<sub>2</sub>; Sigma) was dissolved in ethanol and used at 1 nM concentration. All-*trans*-Retinoic acid (Sigma) was dissolved in DMSO (Sigma) and used at 1  $\mu$ M concentration.

## EMSA

The SOX9RE probe (5'-AGGTCAACAAAG GAGGCAT TGTTTCATCA-3'), the 1/2SOX9RE probe (5'-CAGGTC AACAAAGGAGGA-3'), and a probe containing consensus SOX9 binding site (5'-GATCCGCGCCTTTGTTCTCC CCA-3') [32] were labeled with [ $\gamma$ -<sup>32</sup>P]-ATP (Amersham Biosciences) using T4 Polynucleotide Kinase (USB) and purified using a Sephadex G50 Nick column (Amersham Biosciences). The DNA–protein binding reactions were carried out in a 30  $\mu$ l reaction containing binding buffer (20 mM Hepes–NaOH pH 7.6, 50 mM NaCl, 10 mM DTT, 5% glycerol, 0.5 mM EDTA, and 0.3 mg/ml BSA) and 5  $\mu$ l of in vitro transcribed/translated SOX9 using TNT T7 Quick coupled transcription/translation system (Promega). The reaction was incubated for 15 min incubation at room temperature before adding ~20,000 cpm of the probe followed by 20 min incubation at room temperature. In competition experiments, a 50-fold excess of unlabeled probe was added prior to adding the radiolabeled probe and incubated for 5 min. In supershift experiments, 5  $\mu$ l of anti-SOX9 antibody (H-90, Santa Cruz) was added to the mixture, before adding the probe and incubating for 5 min. The DNA/protein complexes were separated on a 5% native gel in 1 $\times$  TBE. Gels were dried and subjected to phosphor imager analysis.

## qRT-PCR

Low passage MCF-7 cells (ATCC) were grown in phenol red-free DMEM (GIBCO) supplemented with 5% steroid-stripped (charcoal-treated) serum for 72 h prior to addition of atRA or the vehicle (DMSO). Transfection of expression plasmids was done following 72 h growth in steroid-stripped serum. About 1  $\mu$ g of total RNA, extracted from MCF-7 cells using RNeasy (Qiagen), was reverse transcribed into cDNA using Superscript III (Invitrogen) and random hexamers (Amersham). The mRNA expression was measured using Sybr Green (ABI) in an ABI 7500 instrument. About 100 nM of primers for SOX9 was used: Fw (5'-GTACCCGCACTTGACAAAC-3') and Rev (5'-TC GCTCTCGTTCAGAAGTCTC-3') and 300 nM of HES-1 primers: Fw (5'-TAGCTCGCGGCATTCCAAGC-3') and Rev (5'-GTGCTCAGCGCAGCCGTCATCT-3'). An 18S rRNA was used as internal control by using 100 nM of primers: Fw (5'-CCTGCGGCTTTAATTTGACTCA-3') and Rev (5'-AGCTATCAATCTGTCAATCCTGTC-3').

## Western blot

Nuclear proteins were extracted using standard protocols [33] from the MCF-7 cells grown in steroid-stripped serum and phenol-red free DMEM. About 50  $\mu$ g protein per lane was separated on a 12% SDS gel and transferred to a nitrocellulose membrane (Hybond C, Amersham) and exposed to anti-HES-1 [16], anti-SOX9, and anti- $\beta$ -actin antibodies (Santa Cruz). Proteins of interest were visualized using HRP-linked anti-mouse or anti-rabbit IgG (GE Healthcare), Supersignal West Pico chemiluminescent substrate (Pierce), and Hyperfilm ECL (GE Healthcare).

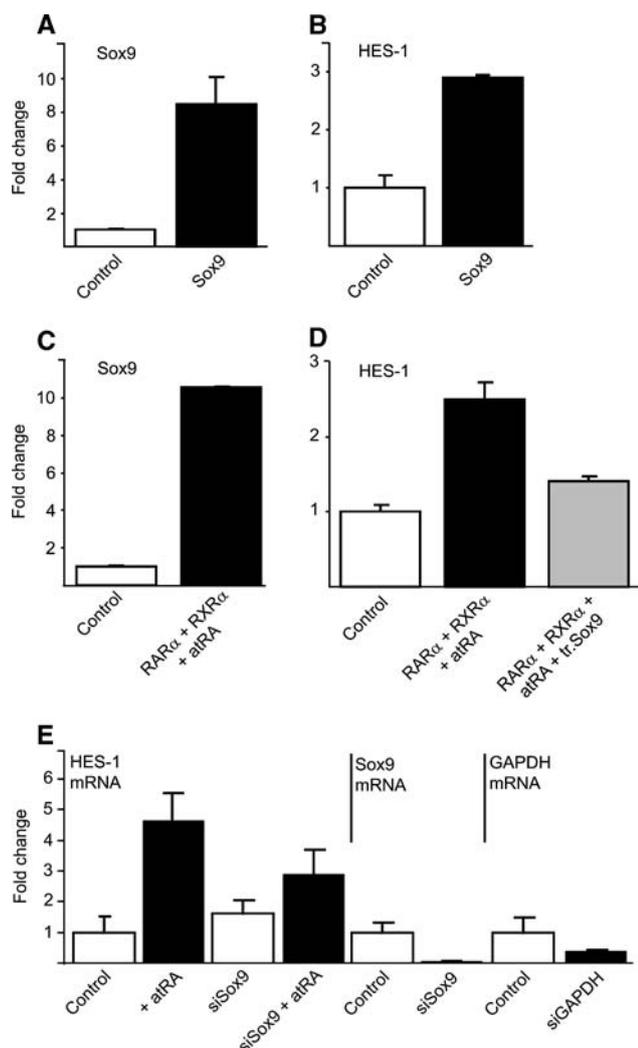
## Chromatin immunoprecipitation

MCF-7 breast cancer cells were starved for 72 h in 25 ml DMEM phenol-red free media supplemented with 5% charcoal filtered FBS. After starvation, the cells were treated with 1  $\mu$ M atRA for 2 h and then prepared for Chromatin immunoprecipitation (ChIP) according to standard ChIP protocols. Immunoprecipitations were done at 4°C overnight using a SOX9 (H-90) antibody from Santa Cruz Biotechnology. DNA was purified using QIAquick<sup>®</sup> PCR Purification kit from Qiagen. Purified DNA was analyzed with quantitative PCR using Roche SYBR Green master mix on a Roche Lightcycler<sup>®</sup> 480. The HES-1 primer sequences for the Q-PCR synthesized by MWG Biotech Inc. are: forward 5'-AGGTTGCAGGTCAACAAAGG-3', and reverse 5'-CTCCAGTCTGCAACCAACA-3'. The input was used to normalize the real-time PCR data.

## Results

Exogenous expression of SOX9 induces HES-1 mRNA expression, and all-*trans* retinoic acid induces mRNA SOX9 expression

We first examined the effect of SOX9 expression on HES-1 levels in MCF-7 cells with an exogenous expression construct of SOX9. Transient transfection with an expression vector containing SOX9 downstream of a CMV promoter enhanced SOX9 mRNA expression eightfold (Fig. 1a). The augmented SOX9 expression resulted in a threefold increase of endogenous HES-1 mRNA expression (Fig. 1b). To determine whether atRA induces endogenous SOX9 mRNA expression in MCF-7 cells, as previously shown [30], we transiently transfected non-confluent MCF-7 cells with RAR $\alpha$  and RXR $\alpha$  plasmids and subsequently added atRA for 6 h prior to harvest. The transfection of RAR $\alpha$  and RXR $\alpha$  plasmids was done to reinforce SOX9 expression in response to atRA. Treatment with atRA resulted in a tenfold increase of SOX9 mRNA expression (Fig. 1c). HES-1



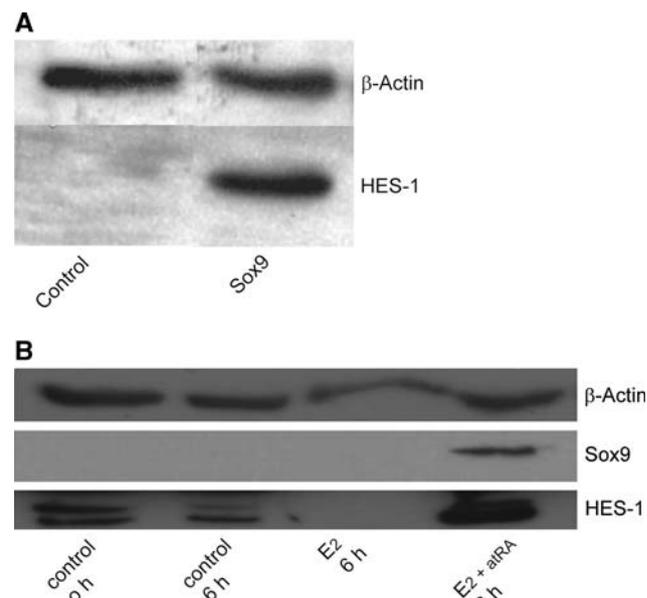
**Fig. 1** HES-1 and SOX9 mRNA expression in MCF-7 cells with exogenous expression of SOX9, or treated with all-*trans* retinoic acid. **a** SOX9 mRNA expression in the transiently transfected MCF-7 cells with SOX9 expression plasmid. **b** HES-1 mRNA expression in MCF-7 cells exogenously expressing SOX9. **c** SOX9 mRNA expression in MCF-7 cells transiently transfected with expression vectors for RAR $\alpha$  and RXR $\alpha$  and treated with atRA for 6 h. **d** Transient transfections of MCF-7 cells with expression vectors for RAR $\alpha$ , RXR $\alpha$ , and truncated SOX9 (tr.SOX9) followed by 6 h treatment with atRA. **e** mRNA expression of HES-1, SOX9, and GAPDH after transfection of siRNA against SOX9. All controls are set to 1. Results are representative of several experiments and presented as fold change from control ( $\pm$ SD)

mRNA expression was induced 2.5-fold in response to atRA (Fig. 1d), as we have shown before [18]. We speculated that the inducible effect on HES-1 mRNA expression by atRA is mediated via SOX9. To examine this possibility, we co-transfected cells with RAR $\alpha$ , RXR $\alpha$ , and a truncated form of SOX9. The truncated SOX9 is postulated to work as a dominant negative SOX9, because it binds to DNA but lacks the transcriptional activation domain [31]. These cells were treated for 6 h with atRA. Approximately 70% of the atRA-induced HES-1 mRNA was suppressed when

truncated SOX9 was expressed or when siRNA against SOX9 was transfected (Fig. 1d, e).

HES-1 protein expression is induced by overexpression of SOX9, and atRA induces SOX9 protein expression

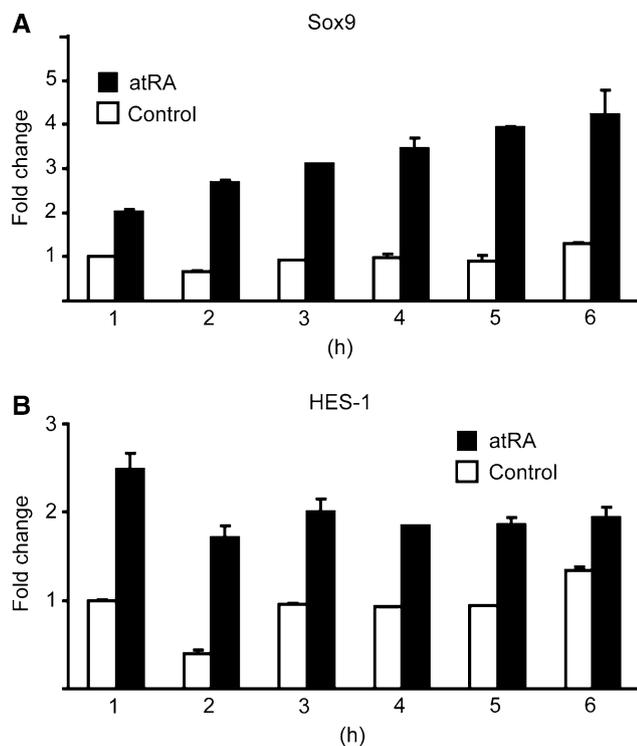
Next, we wanted to determine whether the increase in HES-1 mRNA levels following exogenous SOX9 expression is also reflected in HES-1 protein levels. SOX9 was transfected into MCF-7 cells and 24 h later, the cell nuclei were extracted and subjected to Western blot analysis. HES-1 protein was strongly upregulated by exogenous SOX9 expression (Fig. 2a). We have shown before that HES-1 mRNA and protein expression is downregulated by estrogen [18] and others have shown that both HES-1 and SOX9 are downregulated by estrogen [34]. We therefore treated MCF-7 cells with estrogen to see whether downregulation of HES-1 and SOX9 could be seen in our cells. HES-1 protein was clearly downregulated by 17 $\beta$ -estradiol; however, as SOX9 protein expression in non-induced cells could not be detected, downregulation by estrogen could not be established (Fig. 2b). However, treatment of the same cells with 1  $\mu$ M atRA for 6 h resulted in a robust upregulation of both HES-1 and SOX9 protein. We found no changes in  $\beta$ -actin expression, neither in the presence of estrogen nor in the presence of atRA. These results indicate that atRA induces both SOX9 and HES-1 expression.



**Fig. 2** HES-1 and SOX9 protein expression in MCF-7 cells. Nuclear proteins were harvested and separated on an SDS-gel following exposure to anti-HES-1, anti-SOX9 or anti- $\beta$ -actin antibodies. **a** HES-1 and  $\beta$ -actin expression in cells transfected with SOX9 expression plasmid. Harvest of proteins was performed 24 h after transfection. **b** HES-1, SOX9, and  $\beta$ -actin expression in MCF-7 cells that were grown in the presence or absence of 17 $\beta$ -estradiol (E2) and atRA, as indicated, for 6 h

HES-1 and SOX9 mRNA expression is increased after 1 h in response to atRA and continues to be increased for the following 6 h

It has been shown that HES-1 oscillates in response to serum treatment [35] and it is suggested that HES-1 oscillation is required for efficient cell proliferation [36]. To study the dynamics of SOX9 and HES-1 mRNA expression in response to atRA, we performed a time course study in MCF-7 cells. Non-confluent cells were grown in steroid-depleted serum for 72 h prior to addition of 1  $\mu$ M atRA followed by RNA extraction every hour for 6 h. Relative mRNA expression levels were measured by qRT-PCR. SOX9 mRNA was upregulated twofold as early as after 1 h treatment with atRA (Fig. 3a). We found a steady increase of SOX9 mRNA with time with a maximum fourfold upregulation after 6 h in response to atRA. The non-treated samples expressed SOX9 mRNA at constant levels; the variance from 1 to 6 h was <33% (Fig. 3a). Since we hypothesize that SOX9 regulates HES-1 transcriptionally, we anticipated HES-1 mRNA to be upregulated at the same time points as SOX9 mRNA was, in response to atRA. Indeed, after 1 h in the presence of atRA, HES-1 mRNA was upregulated 2.5 times (Fig. 3b). The steady increase of SOX9 mRNA with time in response to atRA,



**Fig. 3** SOX9 and HES-1 mRNA expression in MCF-7 cells treated with all-*trans* retinoic acid (atRA) for 6 h. MCF-7 cells were grown in steroid-stripped serum for 72 h prior to the addition of 1  $\mu$ M atRA. Cells were harvested every hour for 6 h and subsequently, SOX9 and HES-1 mRNA expression was analyzed using qRT-PCR

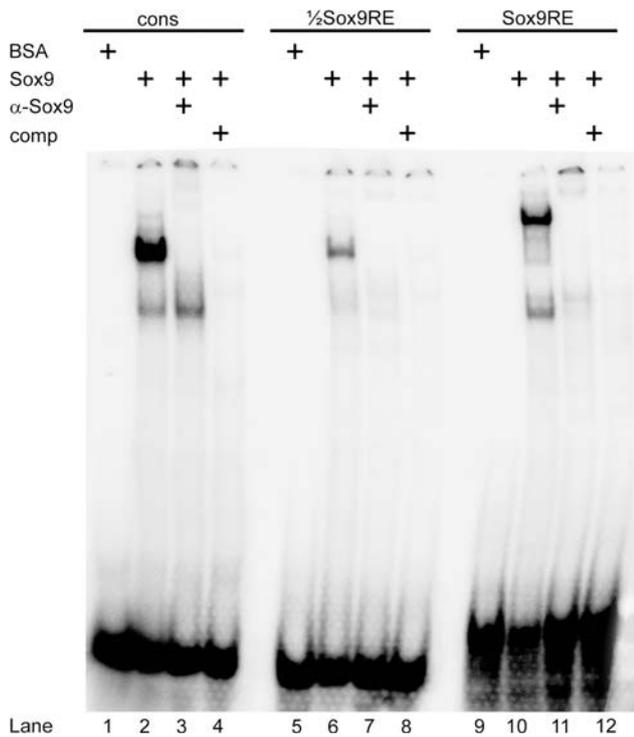
however, was not observed with HES-1 mRNA. Instead we found decreased HES-1 mRNA expression with time, with a minimum of 1.45-fold induction at 6 h treatment with atRA. Furthermore, in contrast to the constant levels of SOX9 mRNA in non-treated samples, we found fluctuating levels of HES-1 mRNA (Fig. 3b).

#### A putative SOX9 response element upstream of the HES-1 gene

To identify a SOX9 response element (SOX9RE) regulating the HES-1 gene, we performed an *in silico* analysis of up to 50 kb upstream of the HES-1 gene. At  $-3,771$  bp 5' of the HES-1 transcriptional start site, we found two putative binding sites for SOX9, arranged in opposite orientation to each other with a 4 bp spacer, AACAA AGgaggCATTGTT. To determine whether this element, containing two putative binding sites for SOX9, could be bound by SOX9 we performed an *in vitro* binding assay. Three double stranded oligonucleotides were designed for the experiment: one probe containing consensus SOX9 binding site (consensus probe) [32], one probe composed of one putative SOX9 binding site ( $\frac{1}{2}$ SOX9RE), and another probe composed of both sites (SOX9RE). We detected very strong binding of *in vitro* transcribed/translated SOX9 to the consensus probe (Fig. 4, lane 2), which was abolished by an anti-SOX9 antibody (lane 3) or by non-consensus probe (lane 4). When we used the probe with one putative SOX9 binding site ( $\frac{1}{2}$ SOX9RE), we detected binding, although weak in comparison to the consensus probe (lane 6). The binding to  $\frac{1}{2}$ SOX9RE was abolished when adding anti-SOX9 antibody (lane 7) or the non-labeled  $\frac{1}{2}$ SOX9RE (lane 8). We detected strong binding to the element that is composed of two putative SOX9 binding sites (SOX9RE; lane 10). The slower migration of the SOX9RE complex compared with consensus probe or  $\frac{1}{2}$ SOX9RE complexes may be explained by the binding of SOX9 to both putative SOX9 binding sites in SOX9RE. The binding of SOX9 to SOX9RE was completely abolished by the non-labeled SOX9RE (lane 12). No super-shift was detected with anti-SOX9 antibody; the complex seems to be immovable in the wells (lanes 3 and 11). Also, we found no binding with 10  $\mu$ g bovine serum albumin (BSA) to any of the probes (lanes 1, 5, and 9). These data show that SOX9 binds, *in vitro*, to the described upstream element in the HES-1 gene.

#### atRA induces SOX9 binding *in vivo* to HES-1 enhancer

To determine whether SOX9 binding to the consensus binding site adjacent to the HES-1 gene occurs *in vivo*, we performed chromatin immunoprecipitation (ChIP) experiments using specific antibodies against SOX9. ChIP

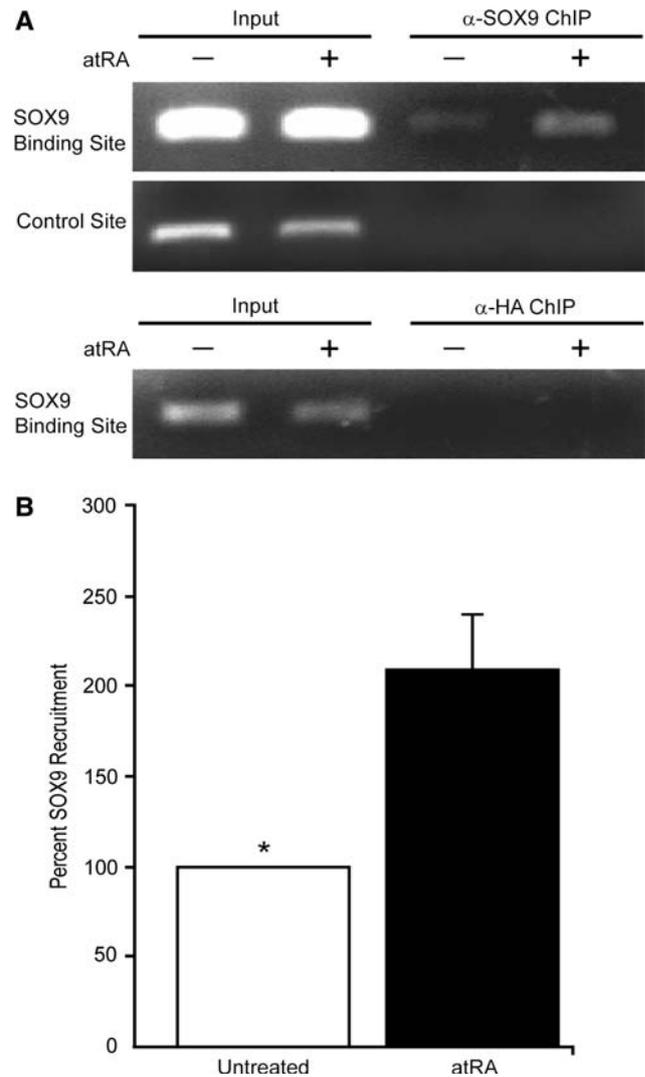


**Fig. 4** SOX9 binding to a putative SOX9 response element upstream of the HES-1 gene as shown by EMSA. In vitro transcribed/translated SOX9 was mixed with either radiolabeled probe containing consensus SOX9 binding site (cons), a probe containing one of the two putative SOX9 binding sites ( $\frac{1}{2}$ SOX9RE) or both putative SOX9 binding sites (SOX9RE). Antibody ( $\alpha$ -SOX9) and non-labeled probe (comp) were added before adding the radiolabeled probe

analysis of SOX9 binding in MCF-7 cells treated with atRA for 2 h showed SOX9 binding to the putative binding site when compared with the untreated controls (Fig. 5a). No SOX9 binding was observed on an irrelevant control site. An average 2.1-fold increase in SOX9 binding was observed by quantitative PCR when compared with untreated controls in three replicate experiments (Fig. 5b). These findings indicate that SOX9 proteins are recruited to the *cis*-regulatory region of HES-1 in response to retinoic acid treatment.

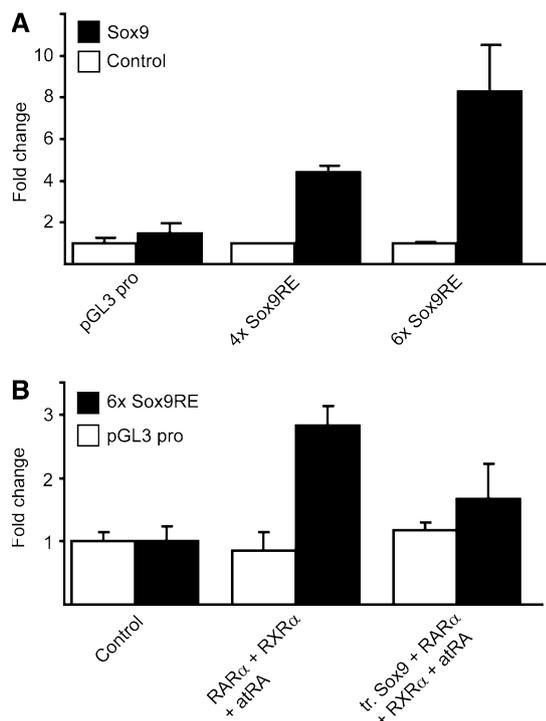
The putative SOX9 response element is transcriptionally active

To examine the transcriptional activity of the consensus SOX9 binding site adjacent to the HES-1 gene, we multimerized the element and cloned it upstream of an SV40 promoter in a luciferase reporter plasmid. We chose to transfect HEK 293 cells rather than MCF-7 cells to accomplish high transfection efficiency. Co-transfections with the reporter construct containing four copies of the element ( $4 \times$  SOX9RE) and the SOX9 expression vector in HEK 293 cells gave a fourfold induction of relative luciferase activity (Fig. 6a). About six copies of the element ( $6 \times$



**Fig. 5** In vivo SOX9 binding to the SOX9 binding site adjacent to the HES-1 gene increases following retinoic acid treatment. **a** Binding of SOX9 to the binding site was visualized by gel electrophoresis of amplicons containing the binding site in the pre-ChIP input and the ChIP samples. MCF-7 cells were treated with  $1 \mu\text{M}$  atRA for 2 h and then subjected to ChIP analysis using specific antibodies against SOX9. Untreated cells were included as a reference control. Experiments using an irrelevant site from the HES1 transcript coding region (Control Site, *central panel*) and antibody against hemmagglutinin (Anti-HA ChIP, *lower panel*) were included as negative controls. **b** SOX9 binding was quantified by real-time PCR and presented as percent binding of the atRA treated samples relative to the untreated controls. The *error bar* represents standard error or means from three independent experiments. Binding of SOX9 in the untreated controls is set at 100% as a reference. Real-time PCR data was normalized to the input

SOX9RE) resulted in an eightfold induction in response to exogenously expressed SOX9. A minor, non-significant change by exogenous expression of SOX9 was seen on the empty vector. To determine whether this enhancer responded to atRA, we co-transfected the reporter,  $6 \times$  SOX9RE, with RAR $\alpha$  and RXR $\alpha$  expression vectors in the presence of



**Fig. 6** Transient transfection of the putative SOX9 response element upstream of HES-1 gene into HEK 293 cells. **a** 4 $\times$  SOX9RE and 6 $\times$  SOX9RE have four and six copies, respectively, of the putative SOX9 response element from HES-1 upstream of the SV40 promoter. Cells were co-transfected with either empty vector (*white bars*) or 100 ng of SOX9 expression vector (*black bars*). **b** A reporter vector containing six copies of the putative SOX9 response element (6 $\times$  SOX9RE, represented by *black bars*) and empty reporter vector (*white bars*) were co-transfected with RAR $\alpha$  and RXR $\alpha$  expression vectors with or without truncated SOX9 expression vector (tr.SOX9). Treatment with all-*trans* retinoic acid (atRA) was done for 6 h. Results are presented as fold change. The experiment was done in triplicates and the results are presented as mean values ( $\pm$ SD)

atRA for 18 h. We found an induction of approximately threefold in the presence of atRA, whereas no induction was found with the empty vector, pGL3 pro, in response to atRA (Fig. 6b). To determine whether the effect observed to atRA on the 6 $\times$  SOX9RE construct was mediated by SOX9, we co-transfected the reporter, RAR $\alpha$  and RXR $\alpha$  with truncated SOX9 and treated the cells for 18 h with atRA. We found that truncated SOX9 strongly inhibited the ability of atRA to activate the 6 $\times$  SOX9RE reporter construct (Fig. 6b). Altogether, these results suggest that SOX9 is a mediator of atRA-induced HES-1 gene expression.

## Discussion

We show in this paper that, in the human breast cancer cell line MCF-7, atRA induces both SOX9 and HES-1 gene expression. We also show that overexpression of SOX9 induces HES-1 gene expression, and that by inhibiting

endogenous SOX9, by overexpressing a dominant negative SOX9, the effect of atRA on HES-1 expression is decreased. Moreover, we found an enhancer regulating HES-1 gene that binds SOX9, *in vitro* and *in vivo*, and that responds to atRA. By using the dominant negative SOX9, we demonstrated that SOX9 is necessary to mediate the effect of atRA on the HES-1 enhancer.

It is postulated that dimerization of SOX9 is required for chondrogenesis, but not for sex determination [37]. SOX9 binds as a monomer in the regulatory region of the sex-determining gene SF1, whereas SOX9 binds as a dimer to enhancers in genes involved in chondrocyte differentiation, such as Col11a2 and Col9a2 [37]. The novel enhancer found  $-3,771$  bp upstream of the HES-1 gene binds dimeric SOX9; it is composed of two SOX9 binding sites in opposite orientation to each other with a 4-bp spacer, similar to the chondrocyte-specific enhancer elements bound and activated by dimeric SOX9 in the type XI collagen gene Col11a2 [38]. By using the enhancer found 5' of the HES-1 gene in an *in vitro* binding assay, we found weak binding to a probe composed of a single SOX9 binding site compared to the stronger binding to a probe composed of the paired SOX9 binding site, indicating binding of a SOX9 dimer. To add another layer of complexity to retinoic acid regulation of HES-1, we have found a putative RARE upstream of the HES-1 gene (characterization is ongoing). Whether retinoic acid regulates HES-1 expression primarily via SOX9 and/or the RARE, synchronously and/or synergistically, is to date not known.

During chondrogenesis, HES-1, retinoic acid, and SOX9 have been shown to be essential components. Expression of HES-1 is enhanced in chondrocytes during chondrogenesis [39], and a recent report suggests that Notch signaling, where HES-1 is an effector protein, is necessary to initiate chondrogenesis, but has to be turned off in order for a continuation of chondrogenesis [40]. Retinoic acid activates RARs that keep chondroprogenitor cells undifferentiated, whereas in the absence of retinoic acid, SOX9 expression and activity are enhanced followed by differentiation of chondroprogenitor cells into chondroblasts [41]. These data are not concordant with our data from breast cancer cells, where we show that retinoic acid induces SOX9 expression, which in turn induces HES-1 expression, suggesting different roles for these factors in different cellular contexts.

Interestingly, SOX9 regulation of HES-1 has recently been suggested to play a role during organogenesis of the pancreas; HES-1 mRNA was decreased following pancreas-specific inactivation of SOX9 in mouse embryos. The pancreas of these mice showed hypoplasia, as a result of depletion of the progenitor pool as seen in HES-1 knockout mice. In the pancreatic epithelium, HES-1 has been shown to inhibit both endocrine and exocrine

differentiation, keeping the progenitor cells in an undifferentiated cell pool. Similarly, SOX9 maintains the pancreatic progenitor cell pool by stimulating its proliferation and survival [42–44]. HES-1 prevents progenitor cells from exiting the cell cycle by repressing the cell cycle inhibitor p57 [43]. However, retinoic acid has been shown to promote endocrine differentiation. Thus, the development of the pancreas does not seem to be in agreement with the regulation of proliferation of breast cancer cells, with reference to the roles of retinoic acid, HES-1, and SOX9 regulation.

In contrast to chondrogenesis and organogenesis of the pancreas, studies suggest that HES-1 plays a different role than SOX9 in intestinal epithelial cell differentiation. HES-1 is expressed in putative progenitor stem cells in crypt base columnar cells and in lower crypt cells, between and above the Paneth cells, respectively, but not in the Paneth cells [45]. Results from gain of function and loss of function studies of Notch cascade/HES-1 indicate that increased HES-1 expression in progenitor cells results in enterocytes, whereas lack of HES-1 expression differentiate cells into secretory lineage cells, which can further differentiate into Goblet, enteroendocrine, and Paneth cells [44, 46–48]. SOX9 on the other hand, is expressed both in the progenitor cell pool and in Paneth cells, where SOX9 is needed for Paneth cell differentiation. In SOX9 knockout mice, there are no Paneth cells formed although enterocytes, Goblet, and endocrine cells exist, whereas HES-1 knockout mice show premature differentiation of Paneth cells [49–51]. The differences of HES-1 action as an inhibitor of differentiation in pancreatic organogenesis, intestinal epithelial cells, and chondrogenesis and as an inhibitor of proliferation of breast cancer cells, respectively, could be due to Notch-dependent or Notch-independent regulation of HES-1 expression. HES-1 has been shown to be regulated, Notch-independently, by c-Jun N-terminal kinase (JNK) in human endothelial cells [52]. Moreover, in HeLa cells, over expression of HES-1 resulted in repression of cyclin-dependent kinase inhibitor p27<sup>Kip1</sup> followed by a promotion of proliferation, whereas, following cell cycle arrest, over expression of HES-1 in PC12 cells led to upregulation of the cell cycle inhibitor p21 [19, 53]. These data are not in accordance with our data from breast cancer cells; we neither see changes of p21 nor of p27 in breast cancer cell lines MCF-7 and T47D when ectopically expressing HES-1 (data not shown). This suggests that the mechanisms described in this paper are likely to be specific to the regulation of breast cancer cell proliferation.

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