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Up-regulation of the *SOX3* gene expression by retinoic acid: characterization of the novel promoter-response element and the retinoid receptors involved

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Abstract

Sox3/SOX3 gene is considered to be one of the earliest neural markers in vertebrates and it is implicated in the genetic cascades that direct brain formation. We have previously shown that early phases of differentiation and neural induction of NT2/D1 embryonal carcinoma cells by retinoic acid (RA) involve up-regulation of the *SOX3* gene expression. Here, we present identification of a novel positive regulatory promoter element involved in RA-dependent activation of the *SOX3* gene expression in NT2/D1 cells. This element represents a direct repeat 3-like motif that directly interacts with retinoid X receptor (RXR) α in a sequence-specific manner. It is capable of independently mediating the RA effect in a heterologous promoter context and its disruption caused significant reduction of RA/RXR transactivation of the *SOX3* promoter. Furthermore, by using synthetic antagonists of retinoid receptors, we have shown for the first time, that RA-induced *SOX3* gene expression could be significantly down-regulated by the synthetic antagonist of RXR. Also, this data showed that RXRs, but not RA receptors, are mediators of RA effect on the *SOX3* gene up-regulation in NT2/D1 cells. Presented data will be valuable for future investigation of *SOX3* gene expression, not only in NT2/D1 model system, but also in diverse developmental, physiological and pathological settings.

Keywords: NT2/D1, response element, retinoic acid, retinoid receptors, *SOX3*.

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The SOX (sex-determining region Y related high mobility group box) proteins comprise a group of transcription factors that act as key regulators of diverse developmental processes (Kamachi *et al.* 2000). SOX3, together with SOX1 and SOX2, belongs to the SOXB1 subgroup of transcriptional activators (Uchikawa *et al.* 1999). *Sox3/SOX3* is considered to be one of the earliest neural markers in vertebrates, playing the role in specifying neuronal fate (Brunelli *et al.* 2003). Dysfunction of the SOX3 protein disturbs the cellular processes required for cognitive and pituitary development, leading to mental retardation and growth hormone deficiency in humans (Laumonnier *et al.* 2002; Stankiewicz *et al.* 2005; Woods *et al.* 2005).

Retinoic acid (RA), a naturally occurring vitamin A derivative, regulates a broad range of biologic processes, with an essential role in neurodevelopment (Maden 2002). According to the current paradigm, RA predominantly exerts its pleiotropic effects through the two families of nuclear retinoid receptors: RA receptors (RARs; NR1B) and retinoid X receptors (RXRs; NR2B), each containing three different receptor types: α, β, and γ (Mangelsdorf *et al.* 1995; Balmer and Blomhoff 2002). Members of these two receptor families form stable heterodimers that, in response to retinoid binding, modulate the transcription of target genes via *cis*-acting RA response elements (RAREs). The majority of classical RAREs consist of a direct repeat (DR) of two hexameric half-sites with the consensus sequence 5′-PuG(G/T)TCA-3′, most commonly separated by two or five 'spacer' nucleotides (DR-2 or DR-5, respectively) (Laudet and Gronemeyer 2002; Bastien and Rochette-Egly 2004). Also, RXR acts as an obligate heterodimeric partner for a various array of other members of the intracellular receptor superfamily (Laudet and Gronemeyer 2002). Numerous hetero-

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Abbreviations used: DR, direct repeat; EC, embryonal carcinoma; EMSA, electrophoretic mobility shift assay; RA, retinoic acid; RAREs, RA response elements; RARs, retinoic acid receptors; RE, response element; RXRs, retinoid X receptors; SOX, SRY related HMG box; *tsp*, transcriptional start point; VDR, vitamin D receptor; WCL, whole cell lysates.

dimers that contain RXR can recognize distinct types of REs. Configuration of these elements, i.e. the arrangement as well as the spacing between the half-sites, is an important determinant to confer the selectivity and binding specificity of receptors (Glass 1994; Laudet and Gronemeyer 2002).

NT2/D1 is a widely characterized pluripotential embryonal carcinoma (EC) cell line that resembles early embryonic stem cells in morphology, antigen expression patterns, biochemistry, developmental potential, and gene regulation (Andrews 1984, 1998). In the presence of RA, NT2/D1 irreversibly differentiates along the neuronal lineage (Andrews 1984; Lee and Andrews 1986), providing an *in vitro* model system for studying human genes that promote and regulate neural differentiation. Previously, we have shown that early phases of differentiation and neural induction of NT2/D1 cells, which take place within 48 h of RA exposure, involve up-regulation of the *SOX3* gene expression at both mRNA and protein levels (Stevanovic 2003; Mojsin *et al.* 2006). Furthermore, in an attempt to delineate RA-responsive element(s) within the *SOX3* promoter, we have described a few *cis*-regulatory elements. Namely, we have shown that an atypical RA/RXRα RE, located −259 to −154 relative to the transcriptional start point (*tsp*) (Mojsin *et al.* 2006), as well as the multiple CCAAT box control elements (Krstic *et al.* 2007) could be recognized as modulators of RA-induced activation of the *SOX3* gene expression.

In this paper, we have focused on the additional regulatory region, previously implied to be involved in RA responsiveness of the *SOX3* promoter (Nikcevic *et al.* 2006). Thorough analysis of this regulatory region resulted in identification of a novel positive regulatory element that is independently capable of mediating an RA/RXR effect in NT2/D1 cells. Also, the presented data has shown, for the first time, that by using synthetic antagonist of RXR, RA-induced SOX3 expression could be significantly down-regulated.

Materials and methods

Generation of CAT reporter constructs

Constructs 0.4HSCAT5, F20R12CAT5, and F19R30CAT6 have been described earlier (Kovacevic Grujicic *et al.* 2005; Mojsin *et al.* 2006; Nikcevic *et al.* 2006). In order to generate the construct F31R31CAT5, complementary oligonucleotides containing *Hin*dIII or *Xba*I restriction enzyme sites (listed in Table S1) were annealed and 3′ recessed ends were filled by a Klenow fragment. Upon digestion, products were cloned into the unique cloning sites (*Hin*dIII and *Xba*I) of pBLCAT5 vector in front of the Herpes simplex virus-TK promoter.

In order to generate F19R30del and F19R30mut reporter constructs, site-directed mutagenesis has been performed by PCR using F19R30CAT6 (−427 to +286, relative to *tsp*) as a template. F19R30del was generated according to the protocol of the EX-site PCR-based site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) using the F35del and R35del primers (listed in Table S1). F19R30mut was generated according to the protocol of the Quick-Change multi-site-directed mutagenesis kit (Stratagene) with the Pmut16 primer (listed in Table S1). Both constructs were sequenced to confirm that no other mutations occurred during PCR amplification.

Cell culture, transfection, and reporter gene analysis

NT2/D1 cells were maintained as described (Andrews 1984). For each transfection a total of 1.2×10^6 NT2/D1 cells were seeded into a 10 cm dish and 5 μg of various *SOX3* CAT6 reporter constructs, together with 1 μg of pBluescript (Stratagene) or pRS-hRXRα expression vector (Mangelsdorf *et al.* 1990) and 3 μg of pCH110 vector (Amersham Pharmacia Biotech, Piscataway, NJ, USA), were co-transfected using the calcium phosphate precipitation method, as described (Nikcevic *et al.* 2006). Ten micrograms of various *SOX3* CAT5 reporter constructs were transfected together with 2 μg of pBluescript or pRS-hRXRα expression vector and 2 μg of pCH110 vector. On the following day, the cells were induced by 10 μM all-*trans* RA (further in this report referred to as RA) alone or in combination with 1 μM LG101208 (a pan-RXR antagonist) or LG100815 (a pan-RAR antagonist) for 48 h. RA was obtained from Sigma (St Louis, MO, USA), while synthetic retinoids LG101208 and LG100815 were a kind gift from M. D. Leibowitz, Ligand Pharmaceuticals, Inc (San Diego, CA, USA). The pBluescript vector was used to adjust the total amount of DNA, while pCH110 vector, expressing β-gal, was used to normalize for transfection efficiency. β-gal 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. All data has been presented as the mean ± SD of at least three independent experiments. Mean values of relative CAT activities were compared with Student's *t*-test. Statistical analyses were performed using SPSS software (SPSS Inc., Chicago, IL, USA); $p < 0.05$ was considered significant.

Preparation of nuclear extracts and the bacterially expressed human recombinant RXRα protein

Nuclear extracts from induced NT2/D1 cells were prepared following 48 h treatment with RA according to standard procedure (Dignam *et al.* 1983).

Glutathione-S-transferase-RXRα fusion protein was expressed from pGEX-2T-RXRα construct, kind gift from R. M. Evans (Mangelsdorf *et al.* 1991), in *Escherichia coli* (DH5α) and purified following the manufacturer instructions (Amersham Pharmacia Biotech).

Electrophoretic mobility shift assays

Forward oligonucleotides used for generating electrophoretic mobility shift assay (EMSA) probes are listed in Table S1. Probes were generated and labeled as described (Table S1).

For EMSA experiments, 600 ng of recombinant RXRα protein or 2.5 μg of nuclear extracts prepared from RA-induced NT2/D1 cells were incubated with 1 ng of ^{32}P-labeled oligonucleotide probe as described (Mojsin *et al.* 2006). In competition analyses, the 400-fold molar excess of an un-labeled competitor was included in the binding reaction. In the supershift assays, 5 μg of rabbit polyclonal antibodies against RXRα (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-553×), RAR (Santa Cruz Biotechnology; sc-773×) or

vitamin D receptor (VDR) (Santa Cruz Biotechnology; sc-1009×) were added to the reaction mixtures and incubated for 15 min at 24°C before the addition of corresponding probes.

Western blot analysis

Whole cell lysates (WCL) were prepared from, either untreated, or NT2/D1 cells treated with 10 μM RA for 48 h. In addition, RA treatment was performed either in the absence or presence of 1 μM synthetic retinoids: LG101208 or LG100815. RA and synthetic retinoids were dissolved in dimethylsulfoxide. For samples not treated with ligands, the same volume of dimethylsulfoxide was added.

For each WCL approximately 10^7 cells were scraped and twice washed in 1× phosphate-buffered saline (Gibco, Rockville, MD, USA). Cells were lysed for 30 min in 1 mL of cold lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40) containing Protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Cell lysates were centrifuged at 10 000 *g* for 15 min at 4°C. Supernatant was collected and stored at −80°C. Thirty micrograms of each WCL sample has been used for western blot analysis as described (Mojsin *et al.* 2006) with rabbit polyclonal antibodies against SOX3 (H-135) and actin (H-196) (Santa Cruz Biotechnology). Actin was employed to indicate the amounts of proteins used for the analysis.

Results

The F20R12 fragment of the *SOX3* promoter is capable of mediating RA/RXR inducibility

In our previous report (Mojsin *et al.* 2006) we have shown that sequences mediating RA induction of the *SOX3* gene reside within the region −427 to −28 relative to the *tsp* (0.4HS region, Fig. 1). Further, we have implied that the region positioned −114 to −21, designated as F20R12 (Fig. 1) is involved in RA responsiveness of the *SOX3* promoter (Nikcevic *et al.* 2006). In order to analyze this region more precisely, we compared the RA effect on reporter construct F20R12 versus 0.4HS construct in NT2/D1 cells in the absence or presence of the RXRα expression vector. The RXRα was chosen as the representative of retinoid receptors, to serve as a tool in the study of *SOX3* gene responsiveness to RA (Mojsin *et al.* 2006).

As it is shown on the histogram in Fig. 1, upon RA treatment, 0.4HS construct displayed approximately a five-fold or 10-fold increase in CAT activity, in the absence or presence of the RXRα expression vector, respectively. Regarding F20R12 construct, RA treatment solely caused approximately a 1.5-fold increase in CAT activity, while in the presence of RXRα expression vector, approximately a fourfold increase was detected.

Therefore, although the response of F20R12 construct to RA, both in the absence or presence of RXRα, has been reduced compared to 0.4HS, this analysis confirmed the existence of an RA/RXR responsive region within this fragment of the *SOX3* promoter.

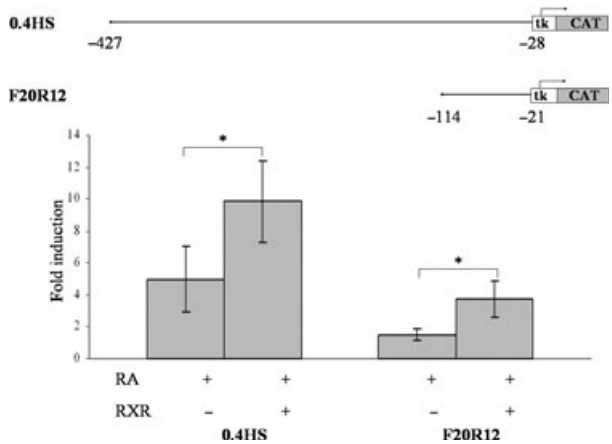

Fig. 1 Analysis of the F20R12 region responsiveness to RA. A schematic illustration of CAT reporter constructs. Numbers represent end points of the genomic fragments relative to the *tsp*. NT2/D1 cells were transfected with, either insert-less pBLCAT5 vector, 0.4HSCAT5 or F20R12CAT5 reporter constructs, in the absence or presence of the RXRα expression vector; untreated or treated with RA. For each construct, fold inductions of CAT activity by RA in the absence or presence of RXRα were normalized by the corresponding pBLCAT5 control values. The mean ± SD of at least four independent experiments is shown. Mean values of relative CAT activities were compared with Student's *t*-test, $*p = 0.01$.

Identification of the RXR-binding site within F20R12 region of the *SOX3* promoter

To explore whether RA stimulation of the F20R12 region is mediated by direct interaction with RXR, EMSAs were performed using recombinant RXRα protein. As shown in Fig. 2, the labeled F20R12 probe binds RXRα, while the addition of the corresponding un-labeled probe almost completely abolishes complex formation, thus confirming the specificity of binding (lanes 2 and 3, respectively). In order to narrow down the RXR binding region within F20R12, competition EMSA has been performed using oligonucleotide probes that encompass the F20R12 region. The presence of a F20R14 or F33R19 probe in competition reactions did not have any effect (lanes 4 and 6, respectively), while the addition of a F31R31 probe diminished complex formation (lane 5). Further, the binding of RXR to F31R31 oligonucleotide was confirmed when the labeled F31R31 probe was used (lane 8). Addition of the corresponding un-labeled probe completely abolished complex formation, demonstrating the specificity of binding (lane 9). Next, we used un-labeled probes, F34R34 or F35R35, that cover F31R31 region, as competitors (lanes 10 and 11, respectively) and showed that only the latter successfully competed the complex formation. Thus, from presented scanning gel shift analysis we concluded that the F20R12 fragment of the *SOX3* promoter contains an RXR binding site that resides within the region 19 bp in size (positioned −71 to −53), designated as F35R35.

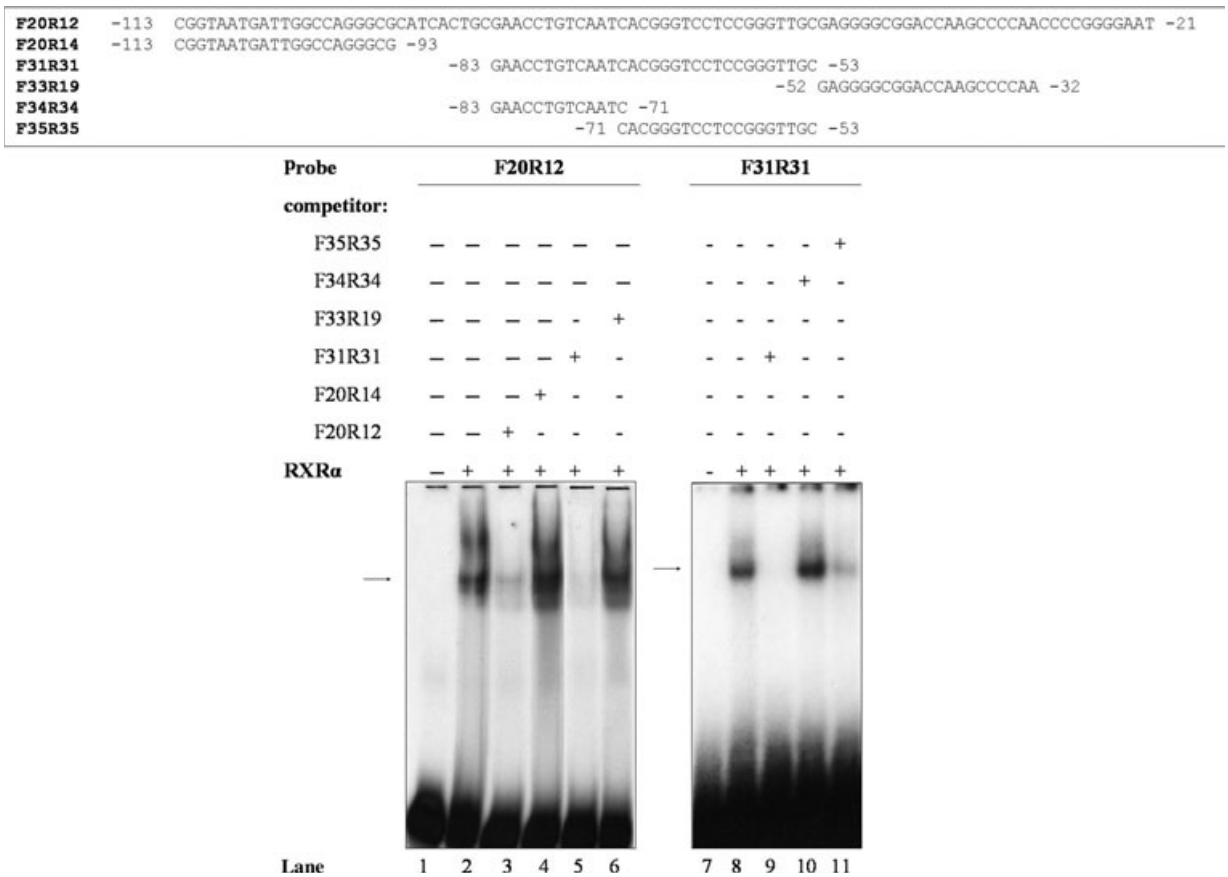

Fig. 2 Scanning gel-shift analysis of the F20R12 probe using the recombinant RXRα protein. Upper panel: nucleotide sequences of probes F20R12, F20R14, F31R31, F33R19, F34R34, and R35R35. EMSAs with radioactively labeled probes F20R12 (lanes 1–6) and F31R31 (lanes 7–11) are shown below. The addition of RXRα and/or particular cold competitors in binding reactions is indicated as +. DNA–protein complexes are indicated by an arrow.

To precisely characterize the RXR binding site within this region, we performed EMSA mutation analysis and compared the RXR binding affinity of *wt* F31R31 to a series of its mutated counterparts (Fig. 3). Mutations were generated within the F35R35 region of the F31R31 probe and the resulting probes were designated as mut1 – mut16 (Fig. 3a). As shown in Fig. 3b, mutations in probes mut3–8, mut11–14 as well as in mut16 significantly abolished RXR binding to these probes, compared to the corresponding *wt* F31R31 probes, thus pointing to the nucleotides that are essential for RXR binding. By this analysis, we identified sequence 5′-**GGGTCC***TCC***GGGTTG**-3′, positioned −68 to −54 within F20R12 region of the *SOX3* promoter, as an RXR binding site. It represents a DR-3-like motif, composed of two half-sites, 6 bp imperfect DRs (represented in bold), separated by 3 bp (italic).

Next, in an attempt to test the binding ability of the described DR-3-like site within the F31R31 probe, we have performed supershift analysis using nuclear extracts from RA-treated NT2/D1 cells (Fig. 4). Firstly, in order to confirm the RXR binding ability of the mapped site, we have co-incubated nuclear extracts with the F31R31 probe or its mutated counterparts in the presence of anti-RXRα antibody. Mutations were generated either to disrupt cores of both half-sites of the described element (mut16), or to fall outside the binding site, to serve as control (mut17) (Fig. 4a). As shown in Fig. 4b, the addition of RXR antibody in reaction with either F31R31 (lane 3) or mut17 (lane 11) resulted in the formation of a supershifted complex. However, when oligonucleotide mut16 was used (lane 7), the supershifted complex could not be detected, confirming that RXRα from nuclear extracts of RA-treated NT2/D1cells interacts with the DR-3-like motif of the *SOX3* promoter in a sequence-specific manner.

Further, in order to test potential RARs involvement in complex formation with F31R31, we applied anti-RAR antibody. We did not detect any supershifted band using F31R31, mut16 or mut17 probe (Fig. 4b, lanes 4, 8, or 12), although the presence of RARs in nuclear extracts has been confirmed using consensus a DR-5 RARE probe from the mouse RARβ promoter (Sucov *et al.* 1990) (Fig. 4b, lane 16).

As the identified DR-3 binding site resembles the configuration of the VDRE (Aranda and Pascual 2001), we tested if

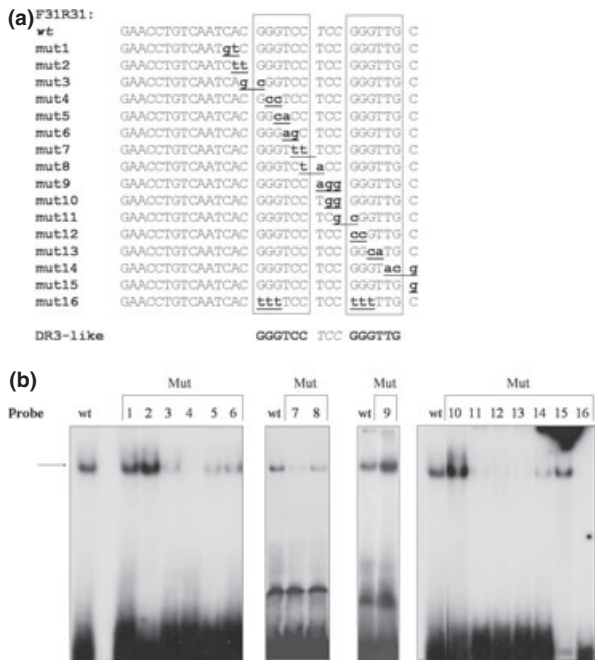

Fig. 3 Identification of RXR binding site within the F31R31 probe by EMSA mutation analysis. (a) Nucleotide sequences of probes: *wt* F31R31 and its mutated counterparts (mut1 – mut16). For each mutant probe, specific residues that were mutated are in lowercase, bold, and underlined. The regions containing the residues whose mutation showed an effect on RXR binding are boxed. The identified DR-3-like motif, composed of two 6 bp imperfect direct repeats (represented in bold) separated by 3 bp (represented in italic) is shown below. (b) Binding of the recombinant RXRα protein to *wt* F31R31 probe and to a series of its mutated counterparts. Radioactively labeled probes that have been used in particular binding reactions are denoted at the top of the corresponding lanes. DNA–protein complexes are indicated by an arrow.

VDR is present in the protein complex formed with the F31R31 probe. As shown in Fig. 4b, lane 20, no supershifted complex has been detected using anti-VDR antibody.

Taken together, we have mapped the DR-3-like site within the F20R12 region of the *SOX3* promoter and have shown that both, recombinant and RXRα from nuclear extracts of RA-treated NT2/D1 cells interact with this site in a sequence-specific manner. Furthermore, neither RAR nor VDR are found to participate in complex formation as RXRs heterodimeric partners.

In the 'native' background RA exerts its effect on the *SOX3* gene expression through the RXRs rather than the RARs family of retinoid receptors

We continued our study of the *SOX3* gene responsiveness to RA in the natural setting. First, it was necessary to determine the involvement of each class of retinoid receptors in RA-induced SOX3 up-regulation on the endogenous level. For that purpose, we used RXRs and RARs pan-antagonists (LG101208 and LG100815, respectively). Results of western blot analysis (Fig. 5a) showed that the RA induction of SOX3 protein expression was reduced in the presence of RXR antagonist (RA + LG101208), while the treatment that included RAR antagonist (RA + LG100815) did not markedly alter the RA effect on SOX3 expression. This result pointed to RXRs, but not RARs, as mediators of the RA effect on the endogenous SOX3 up-regulation in NT2/D1 cells.

Next, in order to provide the 'native' background for the assessment of the DR-3-like element's functional significance in the homologous promoter reporter context, we have chosen a F19R30 construct that harbors an optimal *SOX3* promoter region inserted into the promoter-less reporter vector, pBLCAT6. This regulatory region has been functionally characterized in our previous study (Kovacevic Grujicic *et al.* 2005) and was shown to display responsiveness to RA (Krstic *et al.* 2007). As shown in Fig. 5b, treatment with RA solely, yielded an approximately threefold induction of reporter gene activity (lane 2 vs. 1). Treatment with LG101208 reduced RA responsiveness of the tested construct (lane 3 vs. 2), while treatment with LG100815 did not cause any significant change (lane 4 vs. 2). Further, as expected, the same response was observed when the equivalent experiment was performed in the presence of the RXRα expression vector (Fig. S1). This data has shown that on a selected *SOX3* promoter region the RA effect is also mediated through the RXR family of retinoid receptors.

Taken together, we accurately verified RXRs as major mediators of RA effect on the *SOX3* up-regulation. Also, as the selected *SOX3* promoter reporter construct mirrored the response to synthetic retinoids seen for the endogenous SOX3, we concluded that it represents an adequate model for functional analysis of the newly mapped site.

Newly identified RXR binding site within *SOX3* promoter represents functional response element

In order to test the hypothesis that described DR-3-like RXR binding site represents a RE that mediates RA/RXR inducibility of the *SOX3* promoter, we tested its functional significance using two F19R30 promoter constructs in which this site has been either deleted (*del*) or mutated (*mut*). More precisely, the sequence that was deleted represents the F35R35 region, while the introduced mutation disrupted cores of both half-sites of the putative RE (in concordance with mut16, Figs 3 and 4). As shown in Fig. 6a, RA/RXR responsiveness of *del* and *mut* constructs have been reduced by approximately 50% or 70% compared to the *wt* construct, respectively. This data confirmed that the newly identified RXR binding site could be considered as an RA/RXR RE within the *SOX3* promoter.

Furthermore, we wanted to determine if this RE was able to mediate RA responsiveness in a heterologous promoter context. For that purpose, we cloned F31R31 oligonucleotide into an enhancer dependent reporter vector, pBLCAT5. As

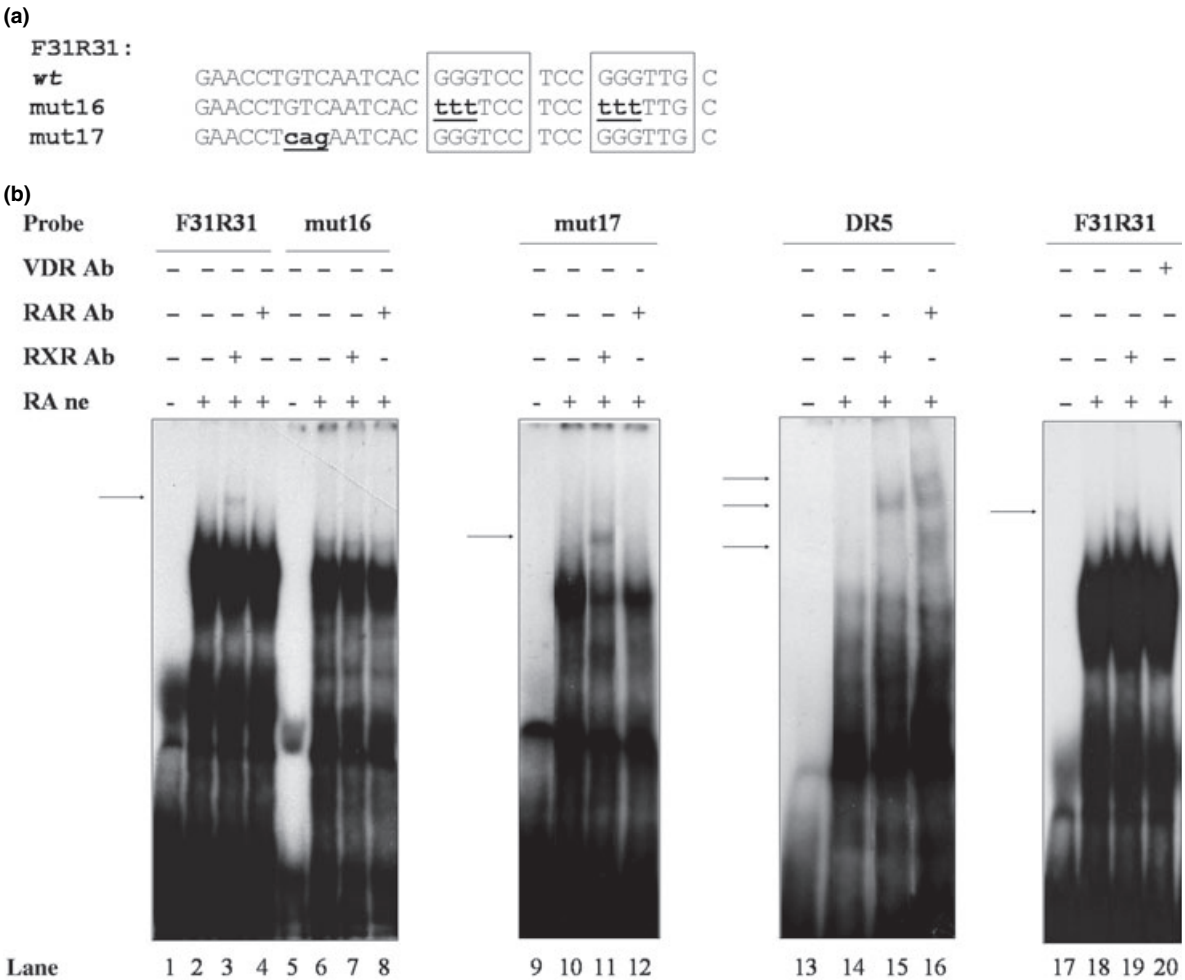

Fig. 4 Characterization of nuclear proteins bound to the DR-3-like site within F31R31 probe. (a) Nucleotide sequences of F31R31 probes: *wt*; mut16 (cores of both half-sites have been mutated); mut17 (mutation outside the DR-3-like binding site). For each mutant probe, specific residues that were mutated are represented in bold and underlined in lowercase. The half-sites of the DR-3-like motif are boxed. (b) Supershift analyses were performed using nuclear extracts from RA-treated NT2/D1 cells (RA ne), specific antibodies (anti-RXRα, anti-RAR and anti-VDR) and particular probes (*wt*, mut16, mut17, and DR-5 probe). DR-5 has been used as a positive control. The addition of RA nuclear extracts and/or a particular antibody in binding reactions is indicated as +, while probes are denoted in the upper row. Supershifted complexes are marked with arrows.

shown in Fig. 6b, RA treatment caused an approximately twofold increase in CAT activity of the F31R31 construct, while an additional fourfold increase was detected in the presence of the RXRα expression vector.

Taken together, this data strongly suggest that the newly identified RXR binding site within *SOX3* promoter represents a functional RE that is independently capable of mediating RA/RXR effect in NT2/D1 cells.

Discussion

Over the years, many RA-regulated genes have been discovered in EC cells that represent a valuable *in vitro* model of early human development. However, precise understanding of the particular gene regulation by retinoids in these cells is yet to be accomplished (Soprano *et al.* 2007). Therefore, the study of the *SOX3* gene expression, for which we have demonstrated that is a direct RA downstream target in NT2/D1 cells, is valuable for future investigation of molecular events underlying EC cells differentiation following RA treatment.

In the present study, we have revealed the existence of an RXR binding motif, a novel positive regulatory element within the *SOX3* promoter. This element represents DR-3 like RE, composed of two imperfect 6 bp DRs. According to its configuration, it might be considered as a VDRE (Aranda and Pascual 2001). However, results of our supershift analysis indicated that VDR should be excluded as an

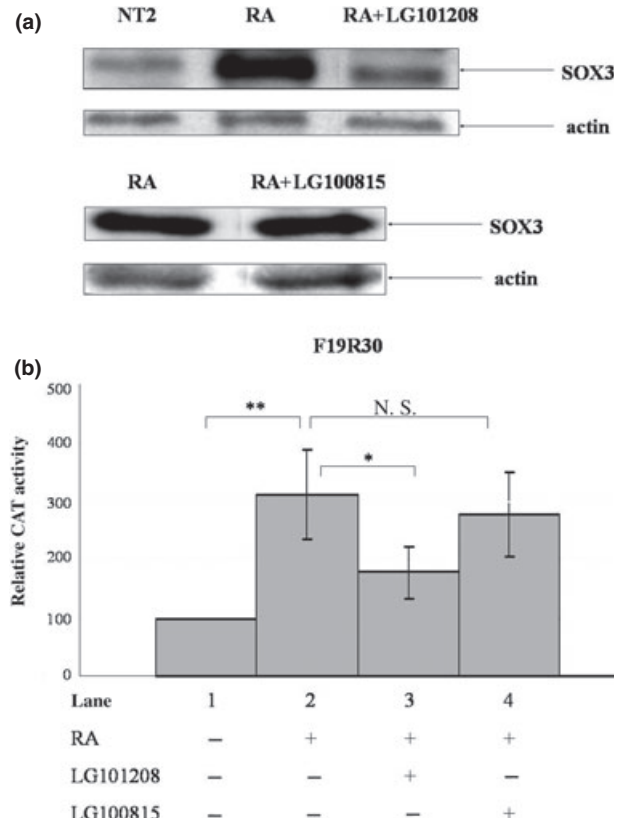

Fig. 5 (a) The effect of RXRs and RARs antagonists on RA-induced SOX3 protein expression in NT2/D1 cells. Western blot analysis of WCL prepared from cells treated for 48 h with: vehicle only (NT2), RA (RA), RA in the presence of RXR antagonist – LG101208 (RA + LG101208) and RA in the presence of RAR antagonist – LG100815 (RA + LG100815). Analyses were performed using antibodies specific for SOX3 and actin that recognize bands of 45 and 43 kDa, respectively, as indicated with arrows on the right. Western blot analyses were performed from at least two independently prepared WCL and one representative blot is presented. (b) Analysis of the response of F19R30 *SOX3* promoter construct to RA in the presence of RXRs and RARs antagonists. NT2/D1 cells were transfected with F19R30 construct and were treated with RA, LG101208 (RXR antagonist) or LG100815 (RAR antagonist), as indicated by +. The normalized CAT activities were calculated as a percentage of the activity of the F19R30 construct in untreated cells, which was set as 100%. Data of at least four independent experiments are presented as the mean ± SD. Mean values of relative CAT activities were compared with Student's *t*-test. $*p < 0.02$, $**p \leq 0.001$, NS, not significant.

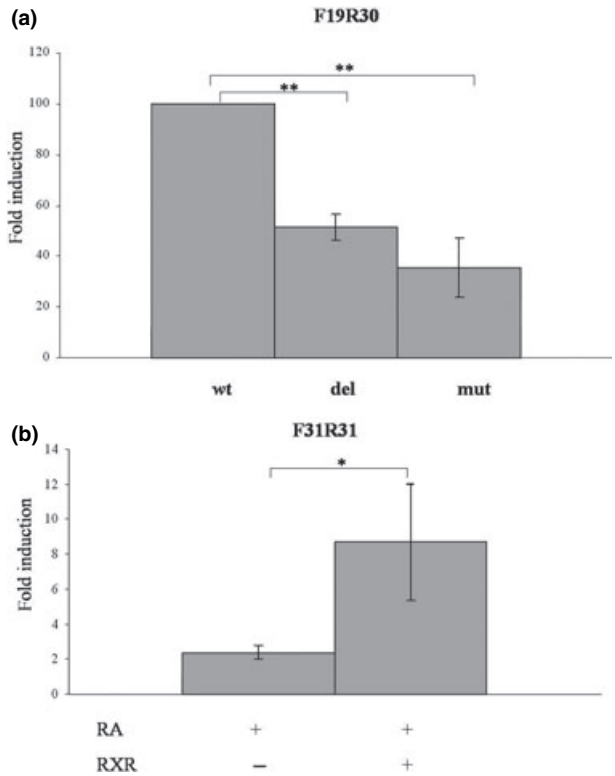

Fig. 6 Analysis of the putative DR-3-like element responsiveness to RA. (a) NT2/D1 cells were co-transfected with RXRα expression vector in the absence or presence of RA, together with either *wt*, *del*, or *mut* F19R30 promoter constructs, as indicated. For each construct fold inductions of CAT activity by RA in the presence of RXRα were calculated and expressed as a percentage of the fold induction of the *wt* F19R30 construct, which was set as 100%. In F19R30 *del* construct, the sequence that represents F35R35 region has been deleted; in F19R30 *mut* construct, cores of both half-sites of DR-3-like element have been mutated. (b) NT2/D1 cells were transfected with, either insert-less pBLCAT5 vector, or F31R31CAT5 reporter construct, in the absence or presence of the RXRα expression vector; untreated or treated with RA. For tested reported construct, fold inductions of CAT activity by RA in the absence or presence of RXRα were normalized by corresponding pBLCAT5 control values. The mean ± SD of at least three independent experiments is shown. Mean values of relative CAT activities were compared with Student's *t*-test. $*p < 0.01$, $**p \leq 0.001$.

heterodimeric partner of RXR acting through this particular binding site (Fig. 4).

Apart from the classical RAREs (Bastien and Rochette-Egly 2004), a number of RAREs with variant core consensus motifs, unusual spacing or symmetry attributes have been reported (Balmer and Blomhoff 2005). Interestingly, this survey pointed out that there are no 'forbidden' nucleotides at any position within RAREs. Bearing this, and the paradigm of RA activity in mind, we suspected potential involvement of RARs with the DR-3-like *SOX3* RE, regardless of its divergence from the prototypical RAREs. Results of our supershift analysis, however, showed that RARs are not involved in attaining RXRs effect through this RE (Fig. 4). Moreover, using the specific RAR antagonist (Fig. 5), we have shown that RA-induced *SOX3* gene expression is predominantly mediated not through RAR, but rather through the RXR family of retinoid receptors.

This is an interesting finding, since for the majority of the genes described it is shown that the direct effect of RA involves mediation by RAR (Laudet and Gronemeyer 2002). However, it is important to point out that among more than

500 genes that have been suggested to be regulatory targets of RA, for only 27 of them it has been unquestionably shown that they are direct targets of the classical RAR–RXR–RARE pathway (Balmer and Blomhoff 2002; Blomhoff and Blomhoff 2006). In many cases, the gene regulation appears to be indirect, reflecting the actions of intermediate transcription factors, non-classical associations of receptors with other proteins, or even more distant mechanisms (Blomhoff and Blomhoff 2006). Recently, it has been confirmed that RXR, independently of RAR, could transduce RA-signaling acting either as homodimer or in heterodimeric complexes (Castillo *et al.* 2004; IJpenberg *et al.* 2004; Szanto *et al.* 2004). Thus, the observed RA effect on *SOX3* gene expression that relies on RXR and not on RAR could be explained in light of these findings. Moreover, exclusion of RARs might indicate integration of RA and another signaling pathway through defined RXR binding site within the *SOX3* promoter. In that view, potential RXR partner whose activity is ligand-dependent would be particularly interesting as it could point out to the specific signaling that together with RA pathway, could be responsible for the fine-tuning of the *SOX3* gene regulation. However, additional experiments, focusing on defining the RXRs homo/hetero dimerization status, are necessary for the precise characterization of this RA–RXR–*SOX3* signaling and its wider biological significance.

Previously, we have reported molecular and functional dissection of the *SOX3* promoter where several *cis*-regulatory elements involved in RA responsiveness were described (Mojsin *et al.* 2006; Krstic *et al.* 2007). In that context, it is interesting to note that functional analyses presented here not only revealed the existence of a novel positive regulatory element, but also implied the presence of potential repressor element(s) within the F20R12 region of the *SOX3* promoter. Namely, F31R31 region displayed higher RA/RXR responsiveness (approximately eightfold, Fig. 6b), compared with the wider, F20R12 region (approximately fourfold, Fig. 1). Thus, the complexity of regulation of the *SOX3* promoter activity, has been additionally underscored by the current study.

The complex system of transcriptional regulation in response to RA has been reported. For instance, within the promoter of the collagen type XI alpha-2 gene an enhancer responsible for chondrocyte-specific expression is itself regulated by liganded RXRβ bound to a downstream DR-4 RARE (Harris *et al.* 2004). Also, it has been reported that RA-induced expression of Burkitt lymphoma receptor 1 gene depends on a RARE that consists of two GT boxes to which RAR and RXR bind, but in a coordinated manner with downstream *cis* elements occupied by octamer-binding transcription factor 1, nuclear factor of activated T cells cytoplasmic calcineurin-dependent 3 and cAMP-RE binding protein 2 factors (Wang and Yen 2004).

In accordance with the reported data, we would like to speculate that accurate expression of the *SOX3* gene during specific stages of development depends on differential usage and/or interplay of the described multiple RAREs within the promoter of this gene. Accordingly, our future work would be focused to define the coordinated action of nuclear receptors, nuclear transcription factor Y and other, not yet identified transcription factor(s), in the up-regulation of *SOX3* gene expression during early stages of neural differentiation of NT2/D1 stem cells.

Further, the results of this study have brought up an interesting concept with potential pharmacological implications. Namely, we have shown that RA activation of the *SOX3* gene expression has been markedly reduced in the presence of pan-antagonist of RXR, LG101208 (Fig. 5). We believe that this could represent valuable information regarding the modulation of *SOX3* gene expression. Also, as a number of links have been found between over-expression of SOX transcription factors and cancers (reviewed in Dong *et al.* 2004), we anticipate that potential pharmacologic interventions that affect the *SOX3* gene expression, including the usage of modulators of RXR activity, might have an influence on tumor progression.

Furthermore, the occurrence of *de novo* neurogenesis in discrete regions of the adult brain and the isolation of neural stem cells from these regions, focused attention on the elucidation of mechanisms involved in neurogenic response in the adult brain (reviewed in Mellough *et al.* 2005). It has been emphasized that the molecular control of neurogenic activity in the adult CNS involves many of the regulatory pathways engaged in the formation of the CNS during embryonic development (Mellough *et al.* 2005). In that respect, the study of developmentally regulated neurogenic genes, such as *Sox* genes, has recently gained additional significance. Actually, several studies suggested that the members of *Sox B1* subfamily continue to be expressed and to maintain neural stem cells in the adult brain (Ferri *et al.* 2004; Ekonomou *et al.* 2005; Wang *et al.* 2006). For *Sox3* gene in particular, it has been shown that is expressed transiently by proliferating and differentiating neural progenitors in the neonatal and adult mouse brain. These findings suggest that Sox3 may continue to regulate mammalian forebrain neural stem/progenitor cell function throughout life (Wang *et al.* 2006).

As the subject of our presented data is RA-dependent regulation of *SOX3* gene expression in NT2/D1 cells, it is worthy highlighting the following. Namely, after exposure to RA, NT2 cells have been used for transplantation as cell therapy for brain injury, ischemia, and neurodegenerative diseases in animal models as well as in two clinical trials of human stroke patients (reviewed in Newman *et al.* 2005). Furthermore, it has been shown that retinoid and retinoid-associated signaling remain active in the adult CNS and that RA-dependent molecular cascade could play a central role in the intrinsic regenerative capacity of the

CNS (reviewed in Malaspina and Michael-Titus 2008). However, despite the recent progress, the replacement of lost cells, either by cell transplantation or by the manipulation of patient's progenitor cells *in situ*, is still not the routine therapeutical practice. In that respect, it is essential to dissect each step of adult neurogenesis in order to enable selection of those mechanisms that could be targets for potential pharmaceutical approaches. We believe that elucidating mechanism(s) underlying regulation of expression of *SOX3* gene in NT2/D1 cells could represent valuable contribution to this field.

To summarize, we have identified a novel positive regulatory element within the *SOX3* promoter that represents a DR-3-like, RXR binding motif, accountable for, at least in part, RA-dependent activation of the *SOX3* gene expression. We have also shown for the first time, to the best of our knowledge, that RA-induced SOX3 expression could be down-regulated by a synthetic compound. In conclusion, our presented data could help, not only to understanding of molecular mechanism(s) responsible for RA-induced *SOX3* gene expression in EC model system, but could also substantially improve the understanding of molecular signals that induce neurogenesis in the stem/progenitor cells under diverse developmental, physiological, and pathological settings.

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Supporting information

Additional supporting information may be found in the online version of this article:

Figure S1. Analysis of the F19R30 *SOX3* promoter construct response to RA in the presence of RXRs and RARs antagonists.

Table S1. Primers used in this study, generation and labeling of probes used in EMSA.

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References

Andrews P. W. (1984) Retinoic acid induces neuronal differentiation of a cloned human embryonal carcinoma cell line *in vitro*. *Dev. Biol.* **103**, 285–293.

Andrews P. W. (1998) Teratocarcinomas and human embryology: pluripotent human EC cell lines. *Review article. APMIS* **106**, 158–167; discussion 167–158.

Aranda A. and Pascual A. (2001) Nuclear hormone receptors and gene expression. *Physiol. Rev.* **81**, 1269–1304.

Balmer J. E. and Blomhoff R. (2002) Gene expression regulation by retinoic acid. *J. Lipid Res.* **43**, 1773–1808.

Balmer J. E. and Blomhoff R. (2005) A robust characterization of retinoic acid response elements based on a comparison of sites in three species. *J. Steroid Biochem. Mol. Biol.* **96**, 347–354.

Bastien J. and Rochette-Egly C. (2004) Nuclear retinoid receptors and the transcription of retinoid-target genes. *Gene* **328**, 1–16.

Blomhoff R. and Blomhoff H. K. (2006) Overview of retinoid metabolism and function. *J. Neurobiol.* **66**, 606–630.

Brunelli S., Silva Casey E., Bell D., Harland R. and Lovell-Badge R. (2003) Expression of Sox3 throughout the developing central nervous system is dependent on the combined action of discrete, evolutionarily conserved regulatory elements. *Genesis* **36**, 12–24.

Castillo A. I., Sanchez-Martinez R., Moreno J. L., Martinez-Iglesias O. A., Palacios D. and Aranda A. (2004) A permissive retinoid X receptor/thyroid hormone receptor heterodimer allows stimulation of prolactin gene transcription by thyroid hormone and 9-cis-retinoic acid. *Mol. Cell. Biol.* **24**, 502–513.

Dignam J. D., Lebovitz R. M. and Roeder R. G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11**, 1475–1489.

Dong C., Wilhelm D. and Koopman P. (2004) Sox genes and cancer. *Cytogenet Genome Res* **105**, 442–447.

Ekonomou A., Kazanis I., Malas S. *et al.* (2005) Neuronal migration and ventral subtype identity in the telencephalon depend on SOX1. *PLoS Biol* **3**, e186.

Ferri A. L., Cavallaro M., Braida D. *et al.* (2004) Sox2 deficiency causes neurodegeneration and impaired neurogenesis in the adult mouse brain. *Development* **131**, 3805–3819.

Glass C. K. (1994) Differential recognition of target genes by nuclear receptor monomers, dimers, and heterodimers. *Endocr. Rev.* **15**, 391–407.

Harris L. R., Kamarainen O. P., Sevakivi M., Miller G. C., Clarke J. W., Potter J. L. and Bridgewater L. C. (2004) A novel retinoic acid-response element requires an enhancer element mediator for transcriptional activation. *Biochem. J.* **383**, 37–43.

IJpenberg A., Tan N. S., Gelman L. *et al.* (2004) *In vivo* activation of PPAR target genes by RXR homodimers. *EMBO J.* **23**, 2083–2091.

Kamachi Y., Uchikawa M. and Kondoh H. (2000) Pairing SOX off: with partners in the regulation of embryonic development. *Trends Genet.* **16**, 182–187.

Kovacevic Grujicic N., Mojsin M., Krstic A. and Stevanovic M. (2005) Functional characterization of the human SOX3 promoter: identification of transcription factors implicated in basal promoter activity. *Gene* **344**, 287–297.

Krstic A., Mojsin M. and Stevanovic M. (2007) Regulation of SOX3 gene expression is driven by multiple NF-Y binding elements. *Arch. Biochem. Biophys.* **467**, 163–173.

Laudet V. and Gronemeyer H. (2002) *The Nuclear Receptor Factsbook*. Academic, San Diego, CA.

Laumonnier F., Ronce N., Hamel B. C. *et al.* (2002) Transcription factor SOX3 is involved in X-linked mental retardation with growth hormone deficiency. *Am. J. Hum. Genet.* **71**, 1450–1455.

Lee V. M. and Andrews P. W. (1986) Differentiation of NTERA-2 clonal human embryonal carcinoma cells into neurons involves the induction of all three neurofilament proteins. *J. Neurosci.* **6**, 514–521.

Maden M. (2002) Retinoid signalling in the development of the central nervous system. *Nat Rev Neurosci* **3**, 843–853.

Malaspina A. and Michael-Titus A. T. (2008) Is the modulation of retinoid and retinoid-associated signaling a future therapeutic strategy in neurological trauma and neurodegeneration? *J. Neurochem.* **104**, 584–595.

Mangelsdorf D. J., Ong E. S., Dyck J. A. and Evans R. M. (1990) Nuclear receptor that identifies a novel retinoic acid response pathway. *Nature* **345**, 224–229.

Mangelsdorf D. J., Umesono K., Kliewer S. A., Borgmeyer U., Ong E. S. and Evans R. M. (1991) A direct repeat in the cellular retinol-binding protein type II gene confers differential regulation by RXR and RAR. *Cell* **66**, 555–561.

Mangelsdorf D. J., Thummel C., Beato M. *et al.* (1995) The nuclear receptor superfamily: the second decade. *Cell* **83**, 835–839.

Mellough C. B., Wood A. and Przyborski S. A. (2005) Pharmacological manipulation of neural progenitor pathways *in situ*: possibilities for neural restoration in the injured adult brain. *Curr. Med. Chem.Cent. Nerv. Syst. Agents* **5**, 67–81.

Mojsin M., Grujicic N. K., Nikcevic G., Krstic A., Savic T. and Stevanovic M. (2006) Mapping of the RXRalpha binding elements involved in retinoic acid induced transcriptional activation of the human SOX3 gene. *Neurosci. Res.* **56**, 409–418.

Newman M. B., Misiuta I., Willing A. E., Zigova T., Karl R. C., Borlongan C. V. and Sanberg P. R. (2005) Tumorigenicity issues of embryonic carcinoma-derived stem cells: relevance to surgical trials using NT2 and hNT neural cells. *Stem Cells Dev.* **14**, 29–43.

Nikcevic G., Kovacevic Grujicic N. and Stevanovic M. (2006) Activation of the HSV-TK promoter in control reporter vector pBLCAT5 by liganded nuclear retinoid receptor RXRalpha. *Arch. Biol. Sci. Belgrade* **58**, 197–203.

Soprano D. R., Teets B. W. and Soprano K. J. (2007) Role of retinoic Acid in the differentiation of embryonal carcinoma and embryonic stem cells. *Vitam. Horm.* **75**, 69–95.

Stankiewicz P., Thiele H., Schlicker M., Cseke-Friedrich A., Bartel-Friedrich S., Yatsenko S. A., Lupski J. R. and Hansmann I. (2005) Duplication of Xq26.2-q27.1, including SOX3, in a mother and daughter with short stature and dyslalia. *Am. J. Med. Genet. A* **138**, 11–17.

Stevanovic M. (2003) Modulation of SOX2 and SOX3 gene expression during differentiation of human neuronal precursor cell line NTERA2. *Mol. Biol. Rep.* **30**, 127–132.

Sucov H. M., Murakami K. K. and Evans R. M. (1990) Characterization of an autoregulated response element in the mouse retinoic acid receptor type beta gene. *Proc. Natl Acad. Sci. USA* **87**, 5392–5396.

Szanto A., Narkar V., Shen Q., Uray I. P., Davies P. J. and Nagy L. (2004) Retinoid X receptors: X-ploring their (patho)physiological functions. *Cell Death Differ.* **11**(Suppl. 2), S126–S143.

Uchikawa M., Kamachi Y. and Kondoh H. (1999) Two distinct subgroups of Group B Sox genes for transcriptional activators and repressors: their expression during embryonic organogenesis of the chicken. *Mech. Dev.* **84**, 103–120.

Wang J. and Yen A. (2004) A novel retinoic acid-responsive element regulates retinoic acid-induced BLR1 expression. *Mol. Cell. Biol.* **24**, 2423–2443.

Wang T. W., Stromberg G. P., Whitney J. T., Brower N. W., Klymkowsky M. W. and Parent J. M. (2006) Sox3 expression identifies neural progenitors in persistent neonatal and adult mouse forebrain germinative zones. *J. Comp. Neurol.* **497**, 88–100.

Woods K. S., Cundall M., Turton J. *et al.* (2005) Over- and underdosage of SOX3 is associated with infundibular hypoplasia and hypopituitarism. *Am. J. Hum. Genet.* **76**, 833–849.

