

## **Regulation of the *SOX3* Gene Expression by Retinoid Receptors**

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### **Short title:**

***SOX3* gene expression by retinoic acid**

## Summary

*Sox3/SOX3* gene is considered to be one of the earliest neural markers in vertebrates. Despite the mounting evidence that *Sox3/SOX3* is one of the key players in the development of the nervous system, limited data are available regarding the transcriptional regulation of its expression. This review is focused on the retinoic acid induced regulation of *SOX3* gene expression, with particular emphasis on the involvement of retinoid receptors. Experiments with human embryonal carcinoma cells identified two response elements involved in retinoic acid/retinoid X receptor-dependent activation of the *SOX3* gene expression: distal atypical retinoic acid-response element, consisting of two unique G-rich boxes separated by 49 bp, and proximal element comprising DR-3-like motif, composed of two imperfect hexameric half-sites. Importantly, the retinoic acid-induced *SOX3* gene expression could be significantly down-regulated by a synthetic antagonist of retinoid receptors. This cell model provides a solid base for further studies on mechanism(s) underlying regulation of expression of *SOX3* gene, which could improve the understanding of molecular signals that induce neurogenesis in the stem/progenitor cells both during development and in adulthood.

**Key words:** *SOX3*, retinoic acid, retinoid receptors, embryonal carcinoma cells, neuronal differentiation,

### ***Sox3/SOX3 gene***

The *Sox/SOX* gene family encodes transcription factors that act as key regulators of diverse developmental processes, such as early embryogenesis, gastrulation, neural induction, formation of various organs and tissues, specification and differentiation of many cell types (Lefebvre *et al.* 2007, Pevny and Lovell-Badge 1997, Wegner 1999). It is known that SOX transcription factors carry a DNA-binding HMG domain and perform their functions in complex interplay with other transcription factors in a manner highly dependent on cell type and promoter context (Kamachi *et al.* 2000). They show both classical and architectural modes of action (Pevny and Lovell-Badge 1997), either activating or repressing specific target genes (Wilson and Koopman 2002). Based on protein sequence comparisons, Sox/SOX transcription factors are divided into 10 distinct groups designated A-J (Bowles *et al.* 2000).

SOX3 together with SOX1 and SOX2 belongs to the SOXB1 subgroup of transcriptional activators (Uchikawa *et al.* 1999), which are pan-neurally expressed and have redundant roles in maintaining the broad developmental potential and identity of neural stem cells (Bylund *et al.* 2003, Pevny and Placzek 2005). In addition to regulating progression of neurogenesis, this group of activators is also operative in post-mitotic neurons (Ekonomou *et al.* 2005, Ferri *et al.* 2004, Rizzoti *et al.* 2004).

*Sox3/SOX3* gene is an X-linked member of the family and the closest relative of sex-determining region Y gene (*SRY*) (Stevanovic *et al.* 1993). It is considered to be one of the earliest neural markers in vertebrates, playing a role in specifying neuronal fate (Brunelli *et al.* 2003). This gene is also implicated in the genetic cascades that direct gonadal development, brain formation, and cognitive function (Pevny and Lovell-Badge

1997, Stevanovic *et al.* 1993, Wegner 1999, Weiss *et al.* 2003). The *Sox3/SOX3* gene is highly expressed in the ventral diencephalon and its deletion is thought to result in either cell death or defects in neuronal activity (Rizzoti *et al.* 2004). Dysfunction of the SOX3 protein disturbs the cellular processes required for cognitive and pituitary developments, leading to mental retardation and growth hormone deficiency in humans (Laumonnier *et al.* 2002, Stankiewicz *et al.* 2005, Woods *et al.* 2005).

Despite the mounting evidence that *Sox3/SOX3* is one of the key players in the development of the nervous system, limited data are available regarding the transcriptional regulation of its expression. Using mouse transgenic reporter assays, Brunelli *et al.* (2003) identified *cis*-regulatory regions of the *mSox3* gene that direct its tissue-specific expression. It has been shown that genomic region comprising 3 kb upstream and 3 kb downstream of the *mSox3* open reading frame, is sufficient to mimic most aspects of the gene's endogenous expression pattern, from the onset of neurogenesis up to midgestation stages (Brunelli *et al.* 2003). The study in *Xenopus laevis* embryos by Rogers *et al.* (2008) indicated that the 1.5 kb upstream regulatory region contains most of the information necessary to recapitulate the spatial and temporal expression of *xSox3* gene. Fine dissection of *xSox3* promoter has revealed the presence of two enhancer regions necessary for full *xSox3* expression, as well as one repression module that restricts the expression of this gene to neuroectoderm (Rogers *et al.* 2008).

Previously we generated data regarding transcriptional regulation of the human *SOX3* gene using embryonal carcinoma (EC) cell line NT2/D1, as a model system. Initially, we determined the *SOX3* transcription start point (*tsp*), promoter region that confers basal promoter activity, as well as positive regulatory elements necessary for

optimal promoter activity (Kovacevic Grujicic *et al.* 2005). Also, we found that ubiquitous transcription factors Sp1 (specificity protein 1), USF1 (upstream stimulatory factor 1), NF-Y (nuclear factor Y), MAZ (*myc*-associated zinc finger protein) and PBX1/MEIS1 (pre-B-cell leukaemia homeobox 1/myeloid ecotropic viral integration site 1 homologue) act as positive regulators of *SOX3* gene expression (Krstic *et al.* 2007, Mojsin and Stevanovic 2010, Kovacevic Grujicic *et al.* 2005, Kovacevic Grujicic *et al.* 2008, Stevanovic 2009). Furthermore, our previous work indicated that retinoic acid (RA) induction of NT2/D1 cells is accompanied by up-regulation of *SOX3* gene expression (Mojsin *et al.* 2006, Stevanovic 2003). This review is focused on the RA-induced regulation of *SOX3* gene expression, with particular emphasis on the involvement of retinoid receptors in this process.

### **NT2/D1 cell line as a model system for neural differentiation**

EC cells are stem cells derived from teratocarcinomas, which arise from transformed germ cells, and are generally considered to be the malignant counterparts of human embryonic stem (ES) cells (Andrews 2002). To date, the most widely characterized EC cell line is NT2/D1. These cells are derived by cloning the NTERA-2 cells established from a nude mouse xenograft of the TERA-2 cell line, which was originally derived from a metastasis of a human testicular teratocarcinoma (Andrews 1984). NT2/D1 cells resemble early ES cells in morphology, antigen expression patterns, biochemistry, developmental potential, and gene regulation (Andrews 1984). Upon culture in RA, NT2/D1 cells differentiate into well developed, morphologic, and immunophenotypic central nervous system-like neurons, with associated loss of cell growth and tumorigenicity (Andrews 1984, Kurie *et al.* 1993, Spinella *et al.* 1999). These

cells have properties similar to those of progenitor cells in the central nervous system (CNS), providing an excellent *in vitro* model system for studying human genes that promote and regulate neural differentiation (Spinella *et al.* 2003).

### **The role of RA in neurodevelopment**

Retinoid signaling contributes to development of the CNS (Maden 2002). In lower vertebrates, it is required for generating the adequate numbers of primary neurons, and for their correct positioning (Sharpe and Goldstone 2000). In the embryonic vertebrate CNS, RA has a role in patterning both the anteroposterior and dorsoventral axes (Cunningham *et al.* 1994). Its main sites of action are the hindbrain and anterior spinal cord (Glover *et al.* 2006). RA also regulates interneuron and motor neuron development along the dorsoventral axis (Sockanathan and Jessell 1998).

Moreover, important regulatory role of RA within the adult CNS has been described (Christie *et al.* 2008). Namely, it was shown that specific areas of the brain, known to undergo active remodeling of neural connections throughout adulthood, synthesize RA (the basal ganglia, olfactory bulbs, hippocampus and auditory afferents) (Dev *et al.* 1993). Importantly, the high levels of RA and associated proteins in the hippocampus, the dentate gyrus in particular, strongly implied the involvement of these molecules in region-specific neurogenesis (Jacobs *et al.* 2006). It was revealed that RA contributed significantly to neuronal differentiation within the dentate gyrus, being involved at a very early stage throughout this process (Jacobs *et al.* 2006).

Deregulation of RA signaling has been suggested to be an underlying cause of several neurological disorders, such as Alzheimer's and Parkinson's diseases (Christie *et al.* 2008). Accordingly, understanding the mechanisms of RA action in the developing

and adult CNS is an important issue, and recently gained additional significance as a therapeutic strategy.

### **RA signaling**

Retinoic acid is naturally occurring, main vitamin A derivative that plays a critical role in the development and homeostasis of all vertebrate tissues, including cell differentiation, proliferation, metabolism and apoptosis (Altucci and Gronemeyer 2001, Ross *et al.* 2000). The retinoid signal is primarily mediated by two families of nuclear retinoid receptors: RA receptors (RARs; NR1B) and retinoid X receptors (RXRs; NR2B), which work as ligand activated transcription factors in a spatio-temporal specific manner (Kastner *et al.* 1997). There are three different RAR and RXR isotypes ( $\alpha$ ,  $\beta$  and  $\gamma$ ) that are encoded by separate genes at distinct chromosomal loci (Chambon 1996). Members of these two retinoid receptor families form stable heterodimers that, in response to RA binding, modulate the transcription of target genes via *cis*-acting RA response elements (REs), RAREs (Mangelsdorf and Evans 1995). The majority of classical RAREs, specifically recognized by RXR/RAR heterodimers, 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) (Bastien and Rochette-Egly 2004, Laudet and Gronemeyer 2002).

In addition, the biological activity of RA is extended by the fact that RXR acts as a homodimer (IJpenberg *et al.* 2004), or as an obligate heterodimeric partner for a various array of other members of the intracellular receptor superfamily, such as thyroid hormone receptors (TRs), vitamin D receptors (VDRs), peroxisome proliferator-activated receptor (PPAR), farnesoid X-activated receptor (FXR), liver-X receptor (LXR) and other (Laudet

and Gronemeyer 2002, Lefebvre *et al.* 2010). These various receptors strongly interact with RXR, and their transcriptional activities are exerted mainly by the resulting heterodimers. RXR thus functions as a “master regulator” of multiple signaling pathways that are essential for mammalian physiology and development (Germain *et al.* 2006). RXR homodimers bind to REs in which the half-sites are separated by 1 bp (DR-1), and RXR containing heterodimers exhibit distinct preferences for a certain spacer length that could be one to five nucleotides (DR-1 to DR-5, respectively) (Laudet and Gronemeyer 2002). Therefore, configuration of these REs, i.e. the arrangement and the spacing between the half-sites, is an important determinant that confers the selectivity and binding specificity of RXR homo/heterodimers (Glass 1994, Laudet and Gronemeyer 2002).

Hundreds of genes have now been shown to be regulated by RA during the processes of neuronal differentiation and neurite outgrowth (Maden 2001). However, only for a minority of them it has been unquestionably shown that 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).

Many RA-regulated genes have been discovered in human EC cells. 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 human *SOX3* gene expression, for which we have shown that is a direct RA downstream target in NT2/D1



EC model system, is valuable for future investigation of molecular events underlying EC cells neuronal differentiation following RA treatment.

### **Localization of RA responsive regions and involvement of retinoid receptors in the regulation of *SOX3* gene expression**

Initially, we showed that *SOX3* gene is expressed in NT2/D1 cells and that early phases of differentiation and neural induction, which take place within 48 h of RA exposure, involve up-regulation of this gene expression at both mRNA and protein levels (Mojsin *et al.* 2006, Stevanovic 2003). This is also in correlation with the literature data based on microarray analysis (Freemantle *et al.* 2002). Since the molecular mechanism(s) underlying regulation of *SOX3* gene by RA are still unknown, our first goal was to search for regions within *SOX3* promoter that mediate RA-induced transcription.

Using enhancer-dependent reporter plasmid approach, we showed that regulatory elements responsible for both, basal and RA-induced transcriptional activation of *SOX3* gene are localized within the 400 bp of its 5'-flanking region (Mojsin *et al.* 2006). To explore whether the *SOX3* induction is mediated by retinoid receptors, overexpression studies with RXR $\alpha$  were employed. The RXR $\alpha$  has been chosen as the representative of retinoid receptors, to serve as a tool in the study of *SOX3* gene responsiveness to RA. Overexpression of RXR $\alpha$  resulted in up-regulation of *SOX3* gene expression in response to RA (Mojsin *et al.* 2006). These results confirmed that RA effect on *SOX3* gene expression is mediated by retinoid receptors.

### **Localization of RA/RXR response elements**

A more systematic search for regulatory regions that are able to mediate responsiveness to RA was performed by deletion mapping of the *SOX3* 400 bp 5'-

flanking region (Mojsin *et al.* 2006, Nikcevic *et al.* 2008). Two response elements involved in RA/RXR-dependent activation of the *SOX3* gene expression were identified (Mojsin *et al.* 2006, Nikcevic *et al.* 2008), designated as distal and proximal REs (Fig. 1).

The distal RA/RXR response element is pinned down to two regulatory elements (Mojsin *et al.* 2006). Only in the presence of both elements, full RA/RXR inducibility is achieved, suggesting they act synergistically. By DNase I footprinting and gel shift analyses, RXR binding motifs were determined, revealing that these regulatory elements comprise two unique G-rich boxes separated by 49 bp (Mojsin *et al.* 2006). Since these motifs did not show any homology to known nuclear receptor REs commonly present in RA-responsive genes, we have proposed it should be considered as a novel, atypical RA/RXR response element (Mojsin *et al.* 2006) (Fig. 1). Interestingly, literature data indicate that RAREs can be increasingly diverse in the core consensus motifs, with unusual spacing or symmetry attributes (Balmer and Blomhoff 2005). For example, activation of the Burkitt lymphoma receptor 1 gene (*blr1*) by RA seems to depend on RARE (17 bp in length), that contains two, arbitrarily named, GT box elements (Wang and Yen 2004). Also, composite response unit that encompasses two degenerate, GC-rich sequences, separated by 30 nucleotides, mediates RA induction of the human retinol-binding protein gene (*RBP*) (Panariello *et al.* 1996).

Further deletion analysis pointed to the presence of an additional, proximal *SOX3* regulatory region responsive to RA that contains an RA/RXR responsive region, as determined by overexpression of RXR $\alpha$  in NT2/D1 (Nikcevic *et al.* 2006, Nikcevic *et al.* 2008). By competition and scanning gel-shift analyses, this region was narrowed down to a 31 bp fragment that directly interacts with recombinant RXR $\alpha$ . Furthermore, by gel-

shift mutation analysis of 31 bp fragment, we identified the sequence 5'-**GGGTCC***tcc***GGGTTG**-3' as an RXR binding site (Nikcevic *et al.* 2008). It represents a DR-3-like motif, composed of two hexameric half-sites, separated by 3 bp (Fig. 1).

In addition, functional significance of the DR-3-like element was indicated by mutational analyses, which showed that disruption/mutation of this element caused significant reduction of RA/RXR transactivation of the *SOX3* promoter (Nikcevic *et al.* 2008). Moreover, the crucial evidence for functional relevance of this regulatory region came from results showing that 31 bp oligonucleotide, encompassing the DR-3-like element, is capable of independently mediating the RA/RXR effect in a heterologous promoter context (Nikcevic *et al.* 2008).

### **Involvement of retinoid receptors in the regulation of *SOX3* gene expression**

Testing the binding ability of the described DR-3-like motif revealed that both recombinant and RXR $\alpha$  from nuclear extracts of RA-treated NT2/D1 cells interact with this motif in a sequence-specific manner. In addition, we have analyzed the potential binding of RARs and VDRs to the DR-3-like *SOX3* RE. The involvement of nuclear receptor VDR was analyzed because the identified DR-3 binding site resembled the configuration of RE characteristic for vitamin D receptors (Aranda and Pascual 2001). However, our experiments suggested that neither RARs nor VDRs participate in complex formation as RXRs heterodimeric partners (Nikcevic *et al.* 2008). This finding emphasized the need to continue study of the *SOX3* gene responsiveness to RA in natural settings, and for that purpose, we have employed synthetic retinoids.

Specifically, advances in the understanding of nuclear receptors at both structural and functional level enabled the design of ligands, selective receptor modulators that

have specific agonist or antagonist features (Altucci *et al.* 2007, de Lera *et al.* 2007). In order to assess the involvement of each class of retinoid receptors in RA-induced SOX3 up-regulation on the endogenous level, we treated NT2/D1 cells with RXRs and RARs pan-antagonists (LG101208 and LG100815, respectively) in the presence of RA (Nikcevic *et al.* 2008). The results of western blot analysis showed that the RA induction of SOX3 protein expression was reduced in the presence of RXR antagonist, whereas the treatment with RAR antagonist did not markedly alter the RA effect on SOX3 expression (Nikcevic *et al.* 2008). This data pointed to RXRs, but not RARs, as mediators of the RA effect on the endogenous SOX3 up-regulation in NT2/D1 cells. It is important to underline that by using this approach, we showed for the first time, that RA-induced SOX3 gene expression could be significantly down-regulated by the synthetic antagonist of RXR.

The observed RA effect on SOX3 gene expression that relies on RXR and not on RAR, might indicate integration of RA and another signaling pathway(s) through defined RXR binding site(s) within the SOX3 promoter. In that view, potential RXR partner(s), whose activity might be ligand dependent, would be particularly interesting implying another signaling that together with RA pathway could be responsible for the fine-tuning of SOX3 gene regulation.

We also studied potential involvement of RXR homodimers in mediating the RA effect on the up-regulation of SOX3 gene expression in NT2/D1 cells. For that purpose, we used a potent and efficacious activator of RXR homodimers (LG100268). Results of western blot analysis showed that RXR homodimers are not responsible for mediating the RA effect on endogenous SOX3 gene expression (Savic *et al.* 2009).

Additional experiments, focusing on defining the RXR partners, are necessary for the precise characterization of this RA–RXR–SOX3 signaling and its wider biological significance.

### **The involvement of other transcription factors in the up-regulation of *SOX3* gene expression by RA**

Our work pointed to multiple CCAAT box control elements within the *SOX3* promoter that could be recognized as modulators of RA-induced activation of *SOX3* gene expression (Krstic *et al.* 2007). Also, we recently showed that the TALE (three-amino-acid loop extension) transcription factors PBX1 and MEIS1 participate in regulating RA-dependent up-regulation of *SOX3* gene expression. It appears that PBX1/MEIS1 directly interact with the binding site within *SOX3* basal promoter region, which is conserved in all analyzed mammalian orthologues, and that these transcription factors are responsible, at least in part, for *SOX3* responsiveness to RA (Mojsin and Stevanovic 2010).

It is also interesting that NF-Y and TR/RXR nuclear receptors functionally interact to confer triiodothyronine (T3)-stimulated transactivation of the hepatic S14 gene (Jump *et al.* 1997). Furthermore, it was reported that PBX1/MEIS1 heterodimers interact with TR/RXR complex to enhance T3 regulation of malic enzyme transcription in hepatocytes (Wang *et al.* 2001).

In accordance with these data, it is reasonable 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, PBX1/MEIS1 and NF-Y binding sites within the promoter of this gene (Fig. 1). Accordingly, further studies are needed to identify the coordinated action of nuclear receptors, NF-Y, PBX1/MEIS1,

and other, not yet identified transcription factor(s), in the up-regulation of *SOX3* gene expression during early stages of RA-induced neural differentiation of NT2/D1 cells.

### **Significance and future directions**

Recently, considerable attention has been focused on understanding the molecular basis of pluripotency and the earliest differentiation processes. The knowledge gained through these studies would pave the path to grow and manipulate pluripotent ES cells efficiently, reproducibly and in a manner appropriate for clinical applications (Johnson *et al.* 2008). NT2/D1 cells are considered as an important alternative to stem cells, having properties similar to those of progenitor cells in the CNS. Therefore, elucidating mechanism(s) underlying regulation of expression of *SOX3* gene in this human EC cells model system will improve the understanding of molecular signals that induce neurogenesis in the stem/progenitor cells both during development and in adulthood. The presented line of study should also accelerate the evaluation of *SOX* genes as potential targets for modulation of proliferation and differentiation of neural progenitors.

Because the neuronal loss is a common feature of many neurological disorders, including stroke, Parkinson's and Alzheimer's diseases, and traumatic brain injury, it is worthy highlighting the following. First, after exposure to RA, NT2 cells were 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 (Newman *et al.* 2005). Second, it was postulated that RA-dependent molecular cascade could play a central role in the intrinsic regenerative capacity of the CNS (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 far

from the routine therapeutic 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 and future clinical applications. We believe that elucidating mechanism(s) underlying regulation of expression of human *SOX3* gene in NT2/D1 cells could represent valuable contribution to this field.

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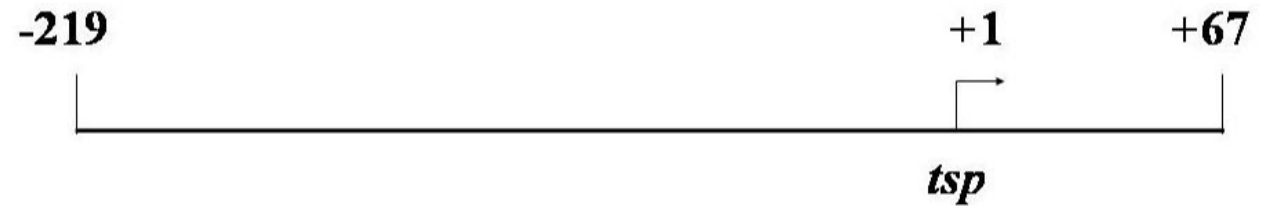
### Figure caption

**Fig. 1. Schematic presentation of the human *SOX3* promoter region, with delineated RXR *cis*-regulatory elements.** Two positive regulatory promoter elements involved in RA/RXR-dependent activation of the human *SOX3* gene expression in NT2/D1 cells were identified: distal RE that consists of two unique G-rich boxes separated by 49 bp, which could be considered as an atypical RA-response element, and proximal RE that represents a DR-3-like motif, composed of two imperfect hexameric DRs, and is characterized as functional RE.

Distal atypical RA/RXR RE: capital letters in bold represent regions that are necessary and sufficient for RXR $\alpha$  binding, lowercase letters mark the extended regions needed for higher binding affinity. Proximal DR-3-like RXR RE: two hexameric halfsites are represented by capital letters in bold, with 3 bp spacer indicated by italic lowercase letters. The binding sites for transcription factors (NF-Y and PBX1/MEIS1) also demonstrated to be involved in the RA-induced up-regulation of *SOX3* gene expression are indicated. All positions are given relative to *tsp*.



## *SOX3* basal promoter region



## 400 bp *SOX3* RA responsive region

