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RETINOIC ACID-INDUCED SOX3 GENE EXPRESSION IN NT2/D1 CELLS IS RXR HOMODIMER-INDEPENDENT

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Abstract — The Sox3/SOX3 gene is implicated in the control of nervous system development. We previously demonstrated modulation of human SOX3 gene expression during neural induction of NT2/D1 cells by retinoic acid (RA). Also, we accurately verified RXR retinoid receptors as major mediators of the effect of RA on SOX3 expression, and excluded RARs as its heterodimeric partners in RA-SOX3 signaling. Here we present evidence that activation of the SOX3 gene by RA is not RXR homodimer-dependent. The described line of SOX3 gene expression studies is valuable for future investigation of the impact that this gene has multiple aspects of normal and pathological development.

Key words: NT2/D1, SOX3 gene, retinoic acid, RXR

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INTRODUCTION

The SOX gene family encodes a highly conserved group of transcription factors that critically control cell fate and differentiation in a multitude of key developmental and physiological processes (reviewed in Lefebvre et al., 2007). 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 HMG box homology, Sox genes are divided into 10 distinct groups designated A-J (Bowles et al., 2000).

Together with SOX1 and SOX2, SOX3 belongs to the SOXB1 subgroup of transcriptional activators (Uchikawa et al., 1999), which are panneurally expressed and have redundant roles in maintaining the broad developmental potential and identity of neural stem cells (reviewed in Lefebvre et al., 2007). In addition to regulating progression of neurogenesis, SOXB1 also functions in post-mitotic neurons (Kiefer, 2007), while SOX3 is an X-linked member of the family (Stevanovic et al., 1993) implicated in the genetic cascades that direct gonadal development, brain formation, and cognitive function (Stevanovic et al., 1993; Pevny and Lovell-Badge, 1997; Wegner, 1999; Weiss et al., 2003). It is considered to be one of the earliest neural markers in vertebrates, playing a role in specifying neuronal fate (Brunelli et al., 2003). Improper SOX3 gene functioning has been linked to severe clinical disorders (Laumonnier et al., 2002; Stankiewicz et al., 2005; Woods et al., 2005).

Retinoic acid (RA) is a naturally occurring vitamin A derivative that regulates a broad range of biological processes, with an essential role in neurodevelopment (Maden, 2002). According to the current paradigm, RA mainly exerts its pleiotropic effects through two families of nuclear retinoid receptors: retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (Mangelsdorf et al., 1995; Balmer and Blomhoff, 2002). In response to retinoid binding, members of these two receptor families form stable heterodimers that modulate transcription of target genes by interacting with *cis*-acting RA response elements (RAREs) (Laudet and Gronemeyer, 2002). It is important to emphasize that RXR can also function as a homodimer or as an

obligatory heterodimeric partner for a diverse array of other ligand-dependent and ligand-independent members of the intracellular receptor superfamily, via distinct types of REs (Laudet and Gronemeyer, 2002). Thus, RXR functions as a "master regulator" of multiple signaling pathways that are essential for mammalian physiology and development (Germain et al., 2006).

To date, the most widely characterized pluripotential embryonal carcinoma (EC) cell line is NT2/D1, which resembles early embryonic stem 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). These cells serve as an *in vitro* model system for studying human genes that promote and regulate neural differentiation (Spinella et al., 2003).

We previously demonstrated that early phases of differentiation and neural induction of NT2/D1 cells by RA involve up-regulation of SOX3 gene expression at both the mRNA and protein levels (Stevanovic, 2003; Mojsin et al., 2006). By using synthetic antagonists of retinoid receptors we accurately verified RXRs as major mediators of RA effect on SOX3 gene up-regulation, i.e., we showed that RXRs, but not RARs, are mediators of the RA effect on SOX3 gene expression (Nikcevic et al., 2008). Another goal of ours was to delineate RA-responsive element(s) within the SOX3 promoter. So far, we have described several such elements, including an atypical RA/RXRα RE, located -259 to -154 relative to the transcriptional start point (*tsp*) (Mojsin et al., 2006), and a DR-3-like RXR RE, positioned -68 to -54 relative to *tsp* (Nikcevic et al., 2008) (Fig. 1).

The goal of the present study was to further assess the dimerization status of the RXR responsible for transducing the RA signal on the SOX3 gene in NT2/D1 cells.

MATERIALS AND METHODS

Cell culture

The NT2/D1 cells used were maintained in Dulbecco's modified Eagle's medium (DMEM, high glucose) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine at 37°C in 10% CO_2. All indicated reagents were obtained from Invitrogen.

Western blot analysis

Whole cell lyzates (WCL) were prepared from either untreated NT2/D1 cells or cells treated for 48 h with 10 μM all-*trans* RA, 1 μM synthetic reti-

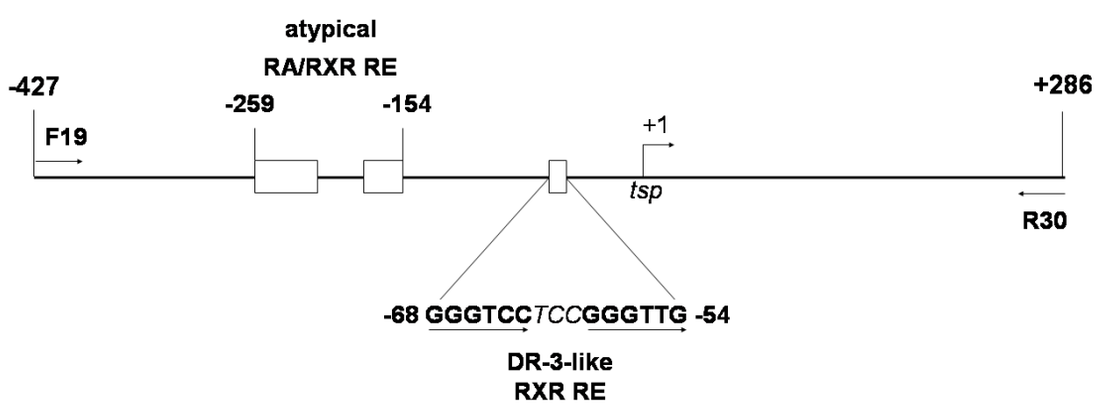

Fig. 1. Schematic illustration of the optimal SOX3 promoter region, with delineated RXR cis-regulatory elements. Numbers represent end points of the promoter fragments relative to the tsp (+1). Arrows at the beginning and end of the depicted region indicate positions of primers used to generate the SOX3 promoter part that was inserted into the pBLCAT6 reporter vector (Kovacevic-Grujicic et al., 2005) and used in subsequent functional analyses.

noids (LG100268, agonist for RXR homodimers, or LG101208, a pan-RXR antagonist), and each synthetic retinoid in combination with RA. The RA and synthetic retinoids were dissolved in dimethylsulfoxide (DMSO). For samples not treated with ligands, the same volume of DMSO was added to the media. We obtained RA from Sigma, while synthetic retinoids were a kind gift from M. D. Leibowitz, Ligand Pharmaceuticals, Inc. (San Diego, CA, USA).

For each WCL, approximately 10^7 cells were scraped and twice washed in 1x phosphate-buffered saline. Cells were lyzed 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 a protease inhibitor cocktail (Roche). Cell lyzates were centrifuged at 10000g for 15 min at 4°C. The supernatant was collected and stored at -80°C. Thirty micrograms of each WCL sample was used for western blot analysis as described previously (Mojsin et al., 2006) with rabbit polyclonal antibodies against SOX3 (H-135) or actin (H-196) (Santa Cruz Biotechnology). Actin was employed to indicate the amounts of proteins used for the analysis.

Transient transfection and reporter gene analysis

The day before transfection, a total of 1.2 x 10^6 NT2/D1 cells were seeded into a 10-cm dish. For each transfection, 5 μg of the SOX3 promoter reporter construct F19R30 CAT6 (Kovacevic-Grujicic et al., 2005) and 3 μg of the pCH110 vector (Amersham Pharmacia Biotech), together with either 1 μg of pBluescript (Stratagene) or the pRShRXRα expression vector (Mangelsdorf et al., 1990), were co-transfected using the calcium phosphate precipitation method, as described elsewhere (Nikčević et al., 2006). On the following day, the cells were either left untreated or treated for 48 h with 10 μM all-*trans* RA, with 1 μM synthetic retinoid LG100268, or with LG100268 together with RA. The pBluescript vector was used to adjust the total amount of DNA, while the pCH110 vector, expressing β-gal, was used to normalize for transfection efficiency. The β-gal and CAT assays were performed as described previously (Kovacevic-Grujicic et al., 2005). Normalized CAT activities were evaluated as a percentage of the promoter reporter construct, which was set as 100% activity. All data are presented as the means ± SD of 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.

RESULTS AND DISCUSSION

We recently demonstrated that the human SOX3 gene is a direct RA downstream target in NT2/D1 cells (Nikcevic et al., 2008). Initial functional *in vivo* analysis revealed that liganded RXRα is a potent activator of endogenous SOX3 protein expression (Mojsin et al., 2006). Next, by applying more precise analysis that included pan-antagonists of RXRs and RARs, we accurately verified RXRs as major mediators of the RA effect on up-regulation of the SOX3 gene (Nikcevic et al., 2008). To be specific, we demonstrated that regardless of the paradigm of RA activity, the RAR family of retinoid receptors are not predominant heterodimeric partners of RXR involved in attaining this RA-RXR-SOX3 signaling (Nikcevic et al., 2008).

Bearing this in mind, and the ability of RXR to bind to cognate DNA and regulate transcription as a homodimer (Mangelsdorf et al., 1991; Zhang et al., 1992; Mascrez et al., 1998; Vivat-Hannah et al., 2003; Ijpenberg et al., 2004), we therefore decided to investigate potential involvement of RXR homodimers in mediating the RA effect on up-regulation of the SOX3 gene. For that purpose, we employed in our study synthetic ligands that selectively bind with high affinity to RXRs: a specific agonist of RXR homodimers (LG100268) and, as a control, an RXR pan-antagonist (LG101208). Results of western blot analysis (Fig. 2 a) showed that treatment of NT2D/1 cells with a potent and efficacious activator of RXR homodimers (LG100268) caused no change in expression of the SOX3 protein level compared to untreated cells (NT2) (lane 3 vs. lane 1). On the other hand, as expected, the presence of RA significantly increased SOX3 expression (RA), while treatment with an RXR pan-antagonist (LG101208) markedly decreased it (lane 2 vs. lane 1 and lane 4 vs. lane 1, respectively). This result was the first indication that, on the endogenous level, RXR homodimers are

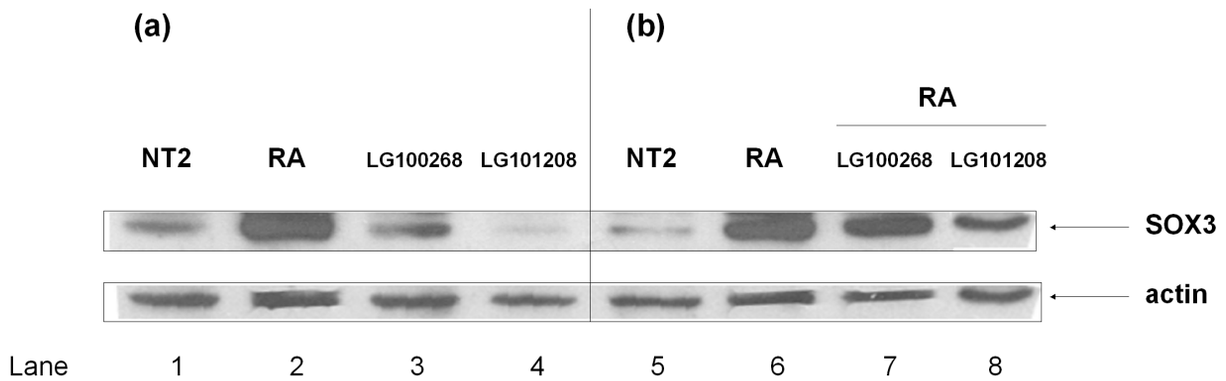

Fig. 2. Effect of RA and RXR specific synthetic ligands on SOX3 protein expression in NT2/D1 cells. (a) and (b) Western blot analysis of WCL prepared from cells treated for 48 h with vehicle only (NT2) or with RA (RA); (a) with an agonist for RXR homodimers (LG100268) or a pan-RXR antagonist (LG101208); (b) with RA in the presence of an agonist for RXR homodimers (RA/LG100268) or RA in the presence of a pan-RXR antagonist (RA/LG101208). Analyses were performed using antibodies specific for SOX3 and actin that recognize bands of 45 and 43 kDa, respectively, as indicated by arrows on the right. Western blot analyses were performed from at least two independently prepared WCL, and one representative blot is presented.

not responsible for mediating the RA effect on SOX3 gene expression.

We next wanted to examine if the addition of an agonist of RXR homodimers together with RA would be able to cause a greater response in SOX3 protein expression than either compound added alone. The result of this analysis is shown in Fig. 2 b. As we indicated earlier, RA-induced SOX3 up-regulation (RA) was reduced in the presence of an RXR antagonist (RA/LG101208) (lanes 6 and 8, respectively), confirming that the NT2/D1 cells used in this set of experiments respond to treatment with ligands for retinoid receptors. However, an agonist of RXR homodimers added together with RA (RA/LG100268) failed to further enhance the RA effect on SOX3 expression (lanes 7 and 6, respectively).

Finally, we decided to test the effect of the synthetic compound LG100268 in the SOX3 promoter reporter context. For that purpose, we selected the F19R30 construct, which harbors an optimal SOX3 promoter region (Fig. 1) inserted into the promoter-less pBLCAT6 reporter vector. This regulatory region was functionally characterized in a previous study of ours (Kovacevic-Grujicic et al., 2005) and was shown to display responsiveness to RA (Krstic et al., 2007), as well as to synthetic antagonists of RXRs and RARs (Nikcevic et al., 2008). As shown in Fig. 3, RA treatment of NT2D/1 cells transfected with the F19R30 construct yielded an approximately threefold induction of reporter gene activity (lane 2 vs. lane 1). Treatment with an agonist of RXR homodimers, both solely (lane 3) and together with RA (lane 4), had no significant effect on SOX3 transcriptional activation (lane 3 vs. lane 1, and lane 4 vs. lane 2), i.e., it mirrored the response seen for endogenous SOX3 (Figs. 2 a and 2 b). Further, an equivalent set of experiments was performed in the presence of a RXRα expression vector (Fig. 3, lanes 5-8). In the presence of RA, approximately 7.5-fold induction of reporter activity was detected (lane 6 vs. lane 5). Again, activation of the F19R30 reporter construct by RA and RXR was not changed in the presence of an agonist of RXR homodimers (lane 8 vs. lane 6). Interestingly, when LG100268 was applied solely in the presence of overexpressed RXRα, significant increase of reporter gene activity was detected compared to untreated cells (lane 7 vs. lane 5), although this activation reached approximately only half of the one caused by RA treatment (lane 7 vs. lane 6). It is important to underline that this effect of LG100268 on SOX3 gene expression was detected only in cotransfection experiments, i.e., when RXRα was overexpressed, and not in the 'native' background (Fig. 2) or under transfection conditions (Fig. 3, lanes 1-4). Nevertheless, the

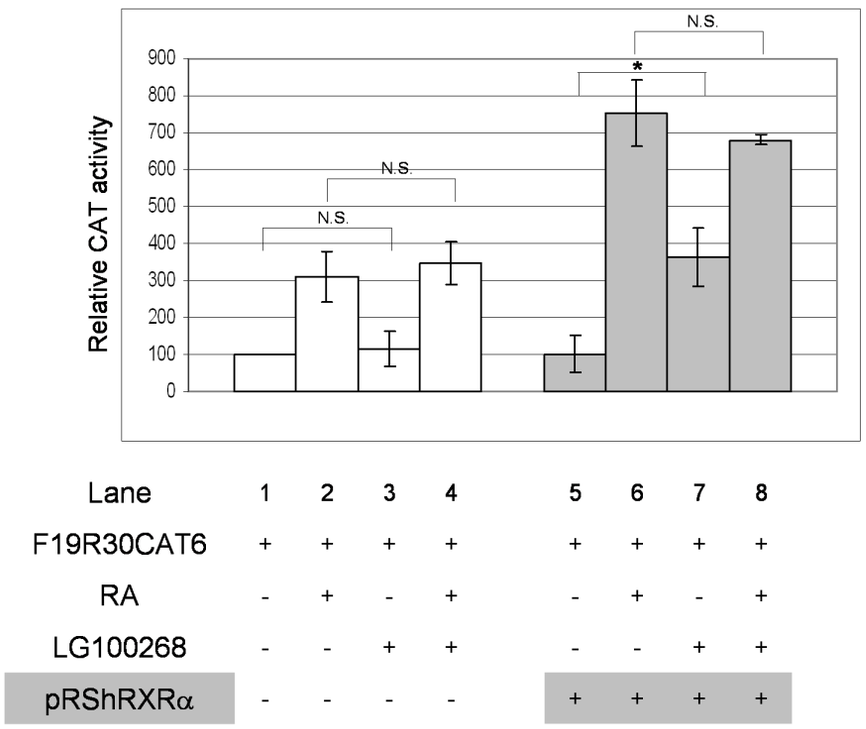

Fig. 3. Analysis of response of the F19R30 SOX3 promoter construct to RA and to a synthetic agonist for RXR homodimers. The NT2/D1 cells were transfected with the F19R30 construct alone (lanes 1-4) or in the presence of the RXRα expression vector (lanes 5-8), and were treated with RA and LG100268, both solely and together, as indicated by +. Normalized CAT activities were calculated as a percentage of activity of the F19R30 construct in untreated cells, which was set as 100%. Data of at least three independent experiments are presented as the means ± SD. Mean values of relative CAT activities were compared with Student's t-test. N.S., not significant, *$p < 0.01$.

detected activation may be related to a characteristic of the LG100268 compound, which, besides acting as a potent activator of RXR homodimers, also acts as an agonist of heterodimers: RXR:PPARs (peroxisome proliferator-activated receptors), RXR:LXRs (liver X receptors), RXR:BAR/FXR (bile acid receptor/farnesoid X receptor), and RXR:NGFI-B (nerve growth factor-induced gene B) (Boehm et al., 1995; Lala et al., 1996; Cesario et al., 2001). We performed MatInspector analysis of the F19R30 SOX3 promoter region and detected the presence of one putative RXR:PPAR heterodimer-binding site (data not shown). It is possible that this element is responsible for the observed activation of the SOX3 promoter by LG100268 in an excess of RXR.

Taken together, the data presented here lead us to conclude that in the natural setting RA-induced SOX3 gene expression is mediated not through RXR acting as a homodimer, but rather through its complexes with other heterodimeric partner(s).

The complexity of transcriptional regulation in response to RA has been widely recognized. In particular, selectivity of the transcriptional response of RXR homo- and heterodimers is a consequence of regulation at multiple levels, such as the availability of cognate ligand(s), dynamics and recruitment of cellular coregulator complexes, and existence of a receptor-specific RE repertoire (that can be highly degenerated) (Khorasanizadeh and Rastinejad, 2001; McKenna and O'Malley, 2002; Vivat-Hannah et al., 2003). In a previous study, we accurately verified RXRs as major mediators of the RA effect on SOX3 expression and showed that RARs can be excluded as heterodimeric partners of RXRs in this RA sig-

naling (Nikcevic et al., 2008). Also, within the SOX3 promoter, we described two RXR *cis*-regulatory elements involved in RA responsiveness (Mojsin et al., 2006; Nikcevic et al., 2008). Here we present evidence that activation of the SOX3 gene by RA is not RXR homodimer-dependent. It is obvious that additional experiments, ones focused on deciphering the hetero-dimerization partner(s) of RXRs, are necessary for the precise characterization of this RA-RXR-SOX3 signaling and its wider biological significance. Moreover, previous results of ours pointed to multiple CCAAT box control elements within the SOX3 promoter that also could be recognized as modulators of RA-induced activation of SOX3 gene expression (Krstic et al., 2007). Altogether, the cited data further underscore complexity of the modulation of SOX3 gene expression by RA.

The important question of how the expression of SOX genes, and the SOX3 gene in particular, is controlled still remains largely unanswered. The described line of SOX3 gene expression studies not only should contribute to understanding molecular events underlying EC cell differentiation, but also be valuable for future investigation of the impact that this gene has on multiple aspects of normal and pathological development.

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ЕКСПРЕСИЈА SOX3 ГЕНА ИНДУКОВАНА РЕТИНОИЧНОМ КИСЕЛИНОМ У NT2/D1 ЋЕЛИЈАМА ЈЕ НЕЗАВИСНА ОД RXR ХОМОДИМЕРА

ТИЈАНА САВИЋ, МИЛЕНА СТЕВАНОВИЋ и ГОРДАНА НИКЧЕВИЋ

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Sox3/SOX3 ген је укључен у контролу развића нервног система. Наша претходна истраживања су показала да током неуралне индукције NT2/D1 ћелија ретиноичном киселином (РК) долази до промене експресије хуманог SOX3 гена. Такође, показали смо да су RXR ретиноидни рецептори главни посредници ефекта РК на SOX3 експресију, док су чланови RAR фамилије рецептора искључени као њихови хетеродимерни партнери у овом сигналном путу. Резултати представљени у овом раду указују да је активација SOX3 гена ретиноичном киселином независна од RXR хомодимера. Ова изучавања експресије SOX3 гена су значајна за будућа истраживања утицаја који овај ген има на различите аспекте нормалног и патолошког развића.