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Establishment and initial characterization of *SOX2*-overexpressing NT2/D1 cell clones

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ABSTRACT. *SOX2*, a universal marker of pluripotent stem cells, is a transcription factor that helps control embryonic development in vertebrates; its expression persists in neural stem/progenitor cells into adulthood. Considering the critical role of the *SOX2* transcription factor in the regulation of genes required for self-renewal and pluripotency of stem cells, we developed and characterized *SOX2*-overexpressing NT2/D1 cell clones. Using Southern blot and semi-quantitative RT-PCR, we confirmed integration and expression of exogenous *SOX2* in three NT2/D1 cell clones. Overexpression of the *SOX2* gene was detected in two of these clones. *SOX2* overexpression in NT2/D1 cell clones resulted in altered expression of key pluripotency genes *OCT4* and *NANOG*. Furthermore, *SOX2*-overexpressing NT2/D1 cell clones entered into retinoic acid-dependent neural differentiation, even when there was elevated *SOX2* expression. After 21 days of induction by retinoic acid, expression of neural markers (*neuroD1* and synaptophysin) was higher in induced cell clones than in induced parental cells. The cell clone with *SOX2* overexpression had an approximately 1.3-fold higher growth rate compared to parental cells. *SOX2* overexpression did not increase the population of cells undergoing apoptosis. Taken together, we developed two *SOX2*-

overexpressing cell clones, with constitutive *SOX2* expression after three weeks of retinoic acid treatment. *SOX2* overexpression resulted in altered expression of pluripotency-related genes, increased proliferation, and altered expression of neural markers after three weeks of retinoic acid treatment.

Key words: *SOX2*; NT2/D1; *OCT4*; *NANOG*; Neural differentiation; Proliferation

INTRODUCTION

To elucidate mechanisms underlying the control of pluripotency/self-renewal of stem cells, and subsequent cell differentiation, several studies have focused on studying the effects of modulation of *Sox2* gene expression in embryonic stem (ES) and embryonal carcinoma (EC) cells (Mitsui et al., 2003; Zhao et al., 2004; Masui et al., 2007; Kopp et al., 2008; Adachi et al., 2010). *Sox2*, a universal marker of pluripotent stem cells, is expressed in the neural tube from the earliest stages of development and is required to maintain neural progenitor cells in a naive state (Graham et al., 2003). Additionally, *Sox2* plays a role in later developmental stages, such as neural differentiation, proliferation and/or maintenance of neural stem cells (Episkopou, 2005) and its expression in the regions of adult neurogenesis is responsible for the maintenance and/or proliferation of adult neural stem cells (Ferri et al., 2004). Studies based on manipulation of *Sox2* gene expression have demonstrated that reduction of *Sox2* in ES cells induces trophoectoderm and endoderm differentiation (Masui et al., 2007; Adachi et al., 2010), while *Sox2* overexpression promotes differentiation into neuroectoderm, mesoderm and trophoectoderm (Zhao et al., 2004; Kopp et al., 2008; Adachi et al., 2010). Furthermore, it was demonstrated that a transient increase in the expression of *Sox2* in murine F9 EC cells reduced the endogenous expression of *Sox2*, *Nanog*, *Oct-3*/*4*, *Fgf-4*, and *Utf1* mRNA (Boer et al., 2007).

NT2/D1 is a widely characterized pluripotent EC cell line that resembles early ES cells in morphology, antigen expression patterns, biochemistry, developmental potential, and gene regulation (Andrews, 1984). In the presence of retinoic acid (RA), NT2/D1 cells differentiate along the neuronal lineage (Andrews, 1984), providing an *in vitro* model system for studying cell differentiation. Moreover, after treatment with RA, NT2 cells have been used for transplantation cell therapy for brain injury, ischemia and neurodegenerative diseases in animal models, as well as in two clinical trials for human stroke patients (reviewed in Newman et al., 2005). Furthermore, it was previously reported that *SOX2* is expressed in NT2/D1 cells and that treatment of these cells with RA is accompanied by significant down-regulation of *SOX2* during early phases of induction (Stevanovic, 2003).

The aim of this study was to modulate *SOX2* expression in order to elucidate the role of this transcription factor in EC NT2/D1 cell pluripotency, proliferation and differentiation. We have shown that increased *SOX2* expression in NT2/D1 cell clones influences expression of key pluripotency genes and the ability of these cells to proliferate. Furthermore, these cell clones differentiate even in the presence of elevated *SOX2* expression. At the same time, treatment with RA for 21 days results in up-regulation of neural markers in *SOX2*-overexpressing cell clones.

MATERIAL AND METHODS

Establishment of a stable *SOX2*-overexpressing NT2/D1 cell line

The pcDNA3.1/*SOX2* construct was obtained by ligation of an *Eco*RI-*Xba*I fragment, containing full-length human *SOX*2 cDNA (Stevanovic et al., 1994), into the *Eco*RI and *Nhe*I sites of expression vector pcDNA3.1. The resulting construct was transfected into NT2/D1 cells using the calcium phosphate precipitation method (Chen and Okayama, 1987). The cells were maintained as previously described (Andrews, 1984). Cells were subjected to drug selection 48 h after transfection by adding G418 (GibcoBRL) to the culture medium (400 µg/mL). G418-resistant, individual colonies were isolated after 3 weeks of culture, with change of the G418-containing medium every 3 days. To assess differentiation potential, NT2/D1 cells and *SOX2*-overexpressing cell clones were grown in media supplemented with 10 µM all-*trans* RA (Sigma) for a period of three weeks.

PCR detection of inserted DNA

In order to detect pcDNA3.1/*SOX2* insertion into the genome, we performed PCR amplification with vector-specific T7 primer 5'-TAA TAC GAC TCA CTA TAG GG-3' (forward) and *SOX2* gene-specific primer 5'-AGA TAC ATG CTG ATC ATG TCC-3' (reverse). Using this primer pair, only template originated from the pcDNA3.1/*SOX2* construct could be amplified (product 860 bp in size).

Southern blot analysis

Integration of pcDNA3.1/*SOX2* into the genome of NT2/D1 cells was verified by Southern blot. Briefly, 20 µg genomic DNA, digested with *Eco*RI restriction enzyme, was fixed on SensiBlot™ Plus Nylon Membrane (Fermentas); membranes were then fixed by UV cross-linking and hybridized with the *SOX2*-specific probe encompassing 3'-coding region.

Reverse transcriptase-PCR (RT-PCR) analysis

Total RNA from untreated or RA-treated NT2/D1 cells and *SOX2*-overexpressing cell clones was isolated using TRI-Reagent (Ambion) according to manufacturer instructions. RNA was treated using a DNA-Free™ kit (Ambion) and subjected to cDNA synthesis. Total RNA (1 µg) was reverse transcribed in the presence of 5 mM $MgCl_2$ (Fermentas), 1X Gold PCR Buffer (Applied Biosystems), 1 mM dNTPs (Pharmacia), 50 U MuLV Reverse Transcriptase (Applied Biosystems), 10 U RNase Inhibitor (Applied Biosystems), and 2.5 µM Random Hexamers (Applied Biosystems). Reactions were carried out at 23°C for 10 min, followed by 42°C for 15 min, denatured at 99°C for 5 min and then cooled to 4°C.

The synthesized cDNAs were amplified with primers specific for both endogenous and exogenous *SOX2*, exogenous *SOX2* only, *OCT4*, *NANOG*, *neuroD1*, synaptophysin, and *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase). Primer sequences for amplification of exogenous *SOX2* were: 5'-ACC AGC TCG CAG ACC TAC AT-3' (forward) and 5'-TCC GCC TCA GAA GCC ATA GA-3' (reverse). Forward primer is from *SOX2*-coding sequence while

reverse primer is from artificial 3'-UTR within the pcDNA3.1, thus enabling amplification of transcript solely originated from pcDNA3.1/*SOX2* construct yielding a 757-bp product. Primer sequences for amplification of overall *SOX2* were: 5'-CCC CTG GCA TGG CTC TTG GC-3' (forward) and 5'-TCG GCG CCG GGG AGA TAC AT-3' (reverse). This primer pair that originated from *SOX2*-coding sequence enables amplification of both exogenous and endogenous *SOX2* yielding a 150-bp product. *OCT4* was amplified with primers: 5'-GCT TTG AGG CTC TGC AGC TT-3' (forward) and 5'-TCT CCA GGT TGC CTC TCA CT-3' (reverse); *NANOG* with primers: 5'-GGT CCC GGT CAA GAA ACA GA-3' (forward) and 5'-TCT GGA ACC AGG TCT TCA CC-3' (reverse); *neuroD1* with primers: 5'-CCA AAA AGA AGA AGA TGA CTA AGG-3' (forward) and 5'-AGC TGT CCA TGG TAC CGT AA-3' (reverse); synaptophysin with primers: 5'-CTT CCT GAA CCT GGT GCT CT-3' (forward) and 5'-TAG CCT TGC TGC CCA TAG TC-3' (reverse); GAPDH with: 5'-GCC TCA AGA TCA TCA GCA ATG C-3' (forward) and 5'-CCA CGA TAC CAA AGT TGT CAT GG-3' (reverse). Expression of GAPDH was used to normalize levels of the total RNA used in assays. PCRs were performed in 20-µL reactions using KAPA 2G Fast HotStart Ready Mix (Kapa Biosystems). All samples were denatured 2 min at 95°C and then cycled at 95°C for 15 s, 60°C for 15 s and 72°C for 15 s, for 35 cycles, with reaction aliquots taken at 25 and 30 cycles. Controls without reverse transcription and without template were included (data not shown).

Western blot analysis

Whole cell lysates were prepared from NT2/D1 and *SOX2*-overexpressing cell clones. Samples were separated by SDS-PAGE on 12% resolving gel and then electrotransferred to nitrocellulose membrane (GE Healthcare). After blocking with 10% nonfat milk at room temperature for 1 h, membranes were incubated overnight at +4°C with the following primary antibodies: rabbit polyclonal antibodies against SOX2 (Abcam, ab15830) and mouse monoclonal antibodies against α-tubulin (Calbiochem, CP06). Immunoreactive bands were detected using an ECL kit (GE Healthcare).

MTT assay

NT2/D1 and *SOX2*-overexpressing cell clones were seeded at a density of 5 x 10^4 cells/well on 96-well plates, and incubated in DMEM containing 10% FCS. At the end of a 24-h culture period, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to cell cultures at a final concentration of 1 mg/mL and cells were incubated for an additional 2 h at 37°C. Subsequently, medium was removed and cells were lysed in DMSO. The conversion of MTT to formazan by metabolically viable cells was monitored by microplate reader at a wavelength of 620 nm. The experiment was done in triplicate and repeated in 12 independent experiments. Cell clones were tested at passages 11-20.

Cell cycle assay

NT2/D1 and *SOX2*-overexpressing cell clones were harvested with Trypsin, washed twice with PBS and fixed in 70% ethanol for 2 h at 4°C. Subsequently, cells were washed with PBS, incubated with RNase (50 µg/mL) 15 min at 37°C and then with 40 µg/mL propidium

iodide (PI) 30 min at 37°C in the dark. Red fluorescence was analyzed on a FACSCalibur flow cytometer (BD Biosciences). Cell distribution among cell cycle phases was determined using the CellQuest Pro software (BD Biosciences). The experiment was performed three times.

RESULTS

Generation and initial characterization of NT2/D1 cells that stably overexpress the *SOX2* gene

In order to establish stable overexpression of the *SOX2* gene in human teratocarcinoma cell line NT2/D1, we cloned full-length human *SOX2* cDNA (Stevanovic et al., 1994) into a pcDNA3.1 vector. Upon transfection of NT2/D1 cell line with a pcDNA3.1/*SOX2* construct and selection using G418, we obtained 54 resistant colonies. Thirty-eight colonies died during propagation and afterwards we established 16 stable cell clones, which were subjected to further analysis.

Using oligonucleotides specific exclusively for *SOX2* originating from a pcDNA3.1/*SOX2* construct we detected the presence of corresponding PCR product of 860 bp in six established cell clones (here referred to as F2, F5, G3, H2, I3, and I4) and by this we have demonstrated that integration occurred in these cell clones (Figure 1A).

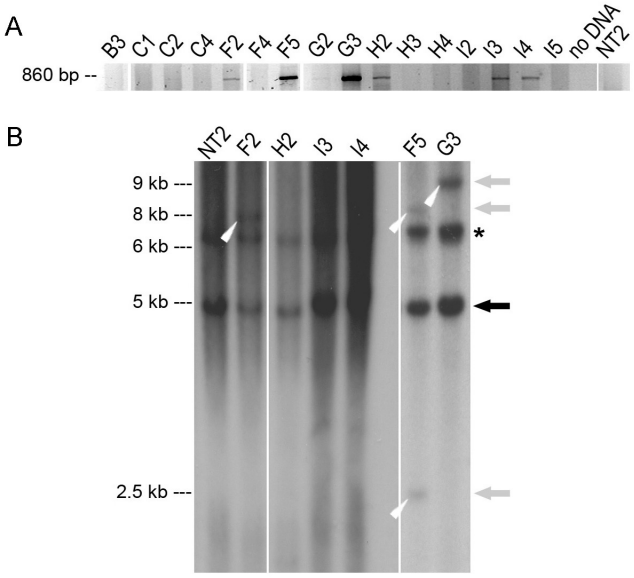


Figure 1. PCR and Southern blot analysis of the NT2/D1 cell clones. **A.** PCR on genomic DNA was performed using primer pairs specific for *SOX2* originating from the pcDNA3.1/*SOX2* construct. A 860-bp product was obtained in cell clones F2, F5, G3, H2, I3, and I4. Control reaction performed on NT2/D1 genomic DNA is presented as NT2; no DNA = negative control. White vertical lines separate merged gel fields. **B.** Southern blot analysis using *SOX2*-specific probe. Integration of the *SOX2* cDNA construct into the genome of F2, F5 and G3 cell clones was detected. Integration was not detected in cell clones H2, I3 and I4. White vertical lines separate merged membrane fields. NT2 = genomic DNA from NT2/D1 cells; Black arrow = endogenous *SOX2*; gray arrows = exogenous *SOX2* size indicator; white arrowheads = exogenous *SOX2*; asterisk = *SOX2* gene-related copy.

To confirm integration of the *SOX2* expression construct, Southern blot was carried out on six previously selected cell clones, as well as on the parental NT2/D1 cell line (Figure 1B). As a result of hybridization with an *SOX2*-specific probe we detected a 5-kb band in all cells analyzed, representing an endogenous *SOX2* gene copy. Southern blot analysis confirmed the integration of the *SOX2* cDNA construct into the genome of F2, F5 and G3 cell clones. We also detected the presence of an additional 2.5-kb band in the F5 cell clone, which is likely the result of integration of fragmented pcDNA3.1/*SOX2* (Figure 1B). Since the integration was not confirmed by Southern blot analysis, cell clones H2, I3 and I4 were excluded from further analysis.

By semi-quantitative RT-PCR analysis, using forward primer specific for the *SOX2*-coding sequence and reverse primer originated from artificial 3'-UTR, we confirmed expression of exogenous *SOX2* in cell clones F2, F5 and G3 (Figure 2A). Relative quantification of exogenous *SOX2* expression did not reveal a statistically significant difference between cell clones F2 and F5, while cell clone G3 has almost three times the expression of exogenous *SOX2*, when compared to F2 (Figure 2A).

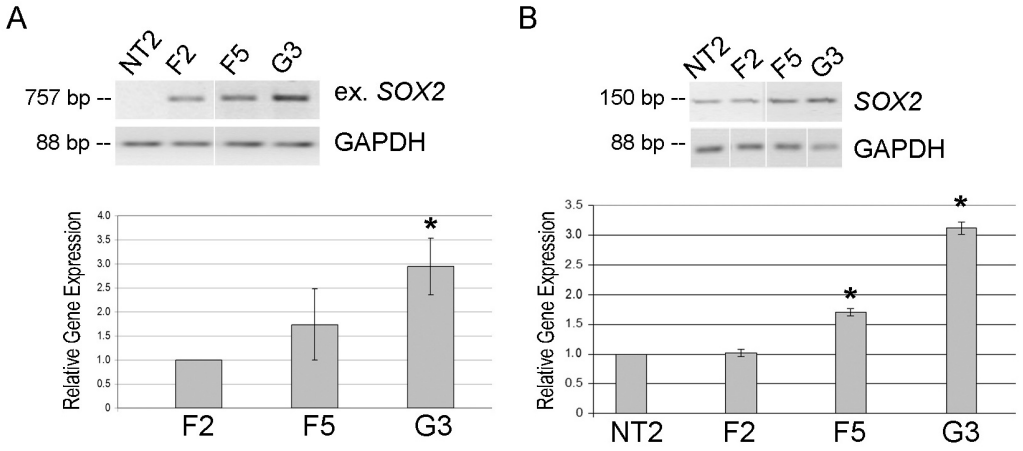


Figure 2. Semi-quantitative RT-PCR analysis of exogenous and overall *SOX2* expression in NT2/D1 cell clones. **A.** RT-PCR analysis of exogenous (ex.) *SOX2* expression in F2, F5 and G3 cell clones. PCR product was not detected in parental NT2/D1 cells that served as control. **B.** RT-PCR analysis of overall *SOX2* expression in F2, F5 and G3 cell clones. *SOX2* expression is increased by approximately 1.7- and 3.1-fold in F5 and G3 cell clones, respectively, while approximately the same level of overall *SOX2* expression is detected in F2 cell clone and NT2/D1 cells. Three independent experiments were performed and representative composite images are shown, with white vertical lines separating merged gel fields. Bands were digitalized, quantified with the ImageJ software and normalized for GAPDH values. The relative gene expression was calculated as a fold expression compared to F2 cell clone (A) or compared to parental NT2/D1 cells (B), which were set as 1. Data of three independent experiments are presented in a histogram as the means ± SD. NT2 = NT2/D1 cells; F2, F5, G3 = NT2/D1 cell clones. *$P \leq 0.001$.

Furthermore, in order to determine overall *SOX2* expression, semi-quantitative RT-PCR analysis was performed using primer pair specific for both endogenous and exogenous *SOX2* (Figure 2B). The overall *SOX2* expression in F5 and G3 cell clones was increased by

approximately 1.7- and 3.1-fold, respectively, when compared to the parental NT2/D1 cells. At the same time approximately the same overall *SOX2* expression was detected in the F2 cell clone and parental cells and accordingly the F2 cell clone was excluded from further analysis.

The overall expression of the SOX2 protein in F5 and G3 cell clones was determined by Western blot analysis (Figure 3). Our results confirm the abundance of the SOX2 protein in NT2/D1 cells, as previously reported (Perrett et al., 2008). Furthermore, our results demonstrate that overall SOX2 protein expression was higher in F5 and G3 cell clones, compared to parental cells.

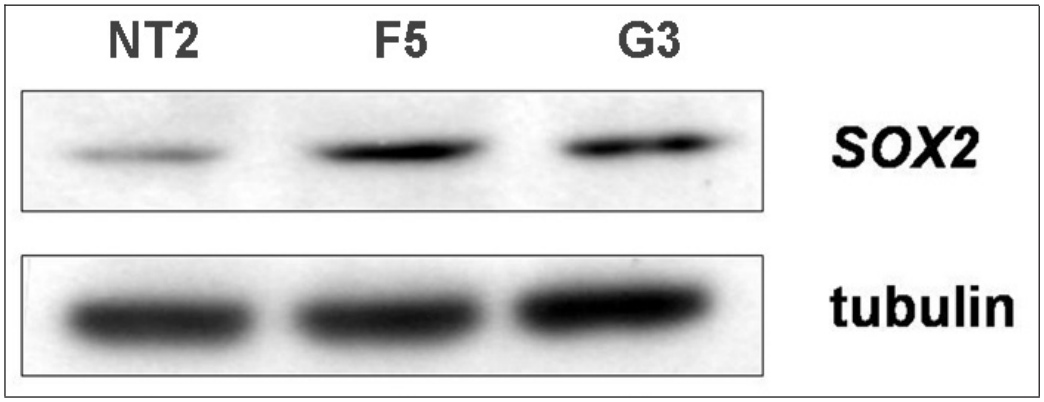


Figure 3. SOX2 protein expression. Protein expression was detected by Western blotting of whole cell lysates using an SOX2 (42 kDa) and α-tubulin (55 kDa)-specific antibody, as indicated on the right. The overall SOX2 protein expression was higher in F5 and G3 cell clones, compared to parental cells. Three independent experiments were performed and one representative blot was shown.

Taken together, our results confirmed the generation of two *SOX2*-overexpressing cell clones that were subjected to further analyses.

Expression profile of pluripotency-specific genes *OCT4* and *NANOG*

Recent studies have demonstrated that transient overexpression of *Sox2* in EC cells results in reduced promoter activity of pluripotency-specific genes, including *Oct4* and *Nanog* (Boer et al., 2007). Furthermore, it has been shown that overexpression of *SOX2* in human ES cells decreases the level of OCT4 and NANOG (Adachi et al., 2010). Therefore, we examined whether *SOX2* overexpression modulates the expression of *OCT4* and *NANOG* in NT2/D1 cells (Figure 4A). In F5 and G3 cell clones, the expression level of the *OCT4* gene was decreased by approximately 2-fold when compared to the parental cell line (Figure 4B). However, *NANOG* expression was down-regulated by approximately one third in the F5 cell clone, while its expression was approximately 1.6-fold higher in the G3 cell clone, compared to the expression in NT2/D1 cells (Figure 4C). Obtained results imply that changes in overall *SOX2* expression in F5 and G3 NT2/D1 cell clones (approximately 1.7 and 3.1 times higher) are able to alter the expression of key pluripotency genes O*CT4* and *NANOG*.

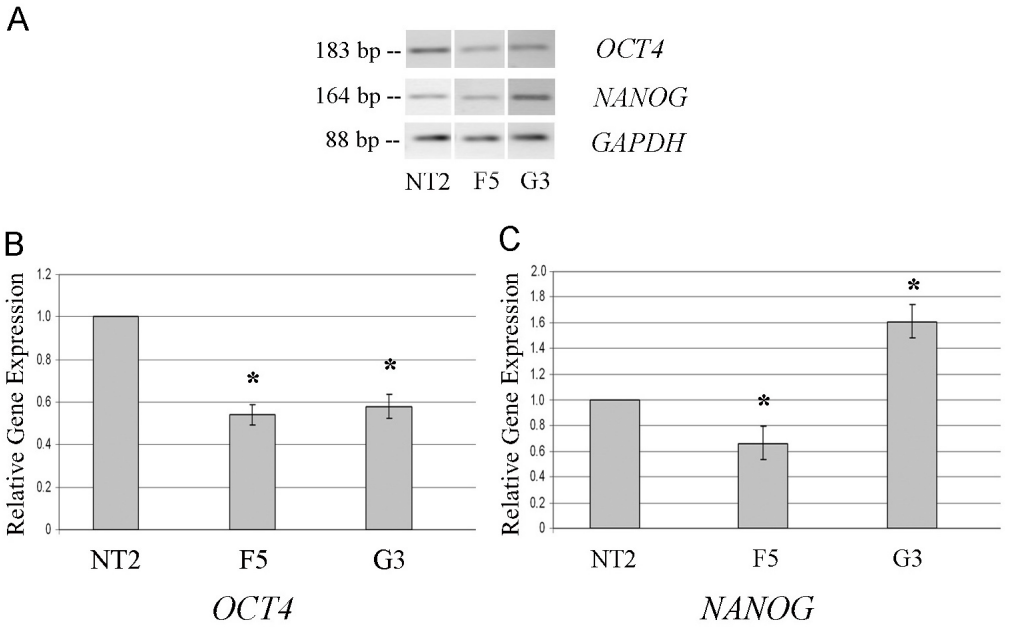


Figure 4. Semi-quantitative RT-PCR analysis of *OCT4* and *NANOG* expression in NT2/D1 cell clones. **A.** RT-PCR analysis of *OCT4* and *NANOG* in cell clones and parental NT2/D1 cells. *OCT4* expression was decreased in F5 and G3 cell clones and *NANOG* expression was decreased in F5 and increased in G3 cell clones, compared with the NT2/D1 cells. Three independent experiments were performed and representative composite images are shown, with white vertical lines separating merged gel fields. Bands were digitalized, quantified with the ImageJ software and normalized for GAPDH values. The relative gene expression of *OCT4* (**B**) and *NANOG* (**C**) were calculated as a fold expression of parental NT2/D1 cells, which were set as 1. Data of three independent experiments are presented in a histogram as the means ± SD. NT2 = NT2/D1 cells; F5, G3 = NT2/D1 cell clones. *$P < 0.05$.

SOX2 overexpression and differentiation

In the presence of RA, NT2/D1 cells differentiate into various cell types, including terminally differentiated neurons and astrocytes (Andrews, 1984; Sandhu et al., 2002). The ability of F5 and G3 cell clones to differentiate was followed by visual inspection during 21 days of culturing cells in media supplemented with RA (Figure 5A). Both cell clones and the parental cell line exerted the ability to grow in highly confluent foci (data not shown). However, a small fraction of the cells exhibited substantially altered morphology and developed cells resembling neurons (Figure 5A). Considering data that endogenous *SOX2* expression is significantly down-regulated in NT2/D1 cells upon induction with RA (Stevanovic, 2003), our next goal was to determine whether cell clones F5 and G3 retain *SOX2* expression after 21 days of induction by RA. Relative quantification of overall *SOX2* expression in NT2/D1 cells and F5 and G3 cell clones was assessed after three weeks of treatment with RA (Figure 5B-D). As expected, *SOX2* expression in NT2/D1 cells was down-regulated by 2-fold when compared to their uninduced counterpart cells (Figure 5D). The analysis revealed that cell clones retained statistically significant *SOX2* overexpression (approximately 1.4- and 2.3-fold increase

in F5 and G3 cell clones, respectively) compared to NT2/D1 cells (Figure 5C). Interestingly, overall *SOX2* expression in the G3 cell clone treated with RA for 21 days is approximately 1.3-fold higher when compared with uninduced parental NT2/D1 cells (Figure 5D).

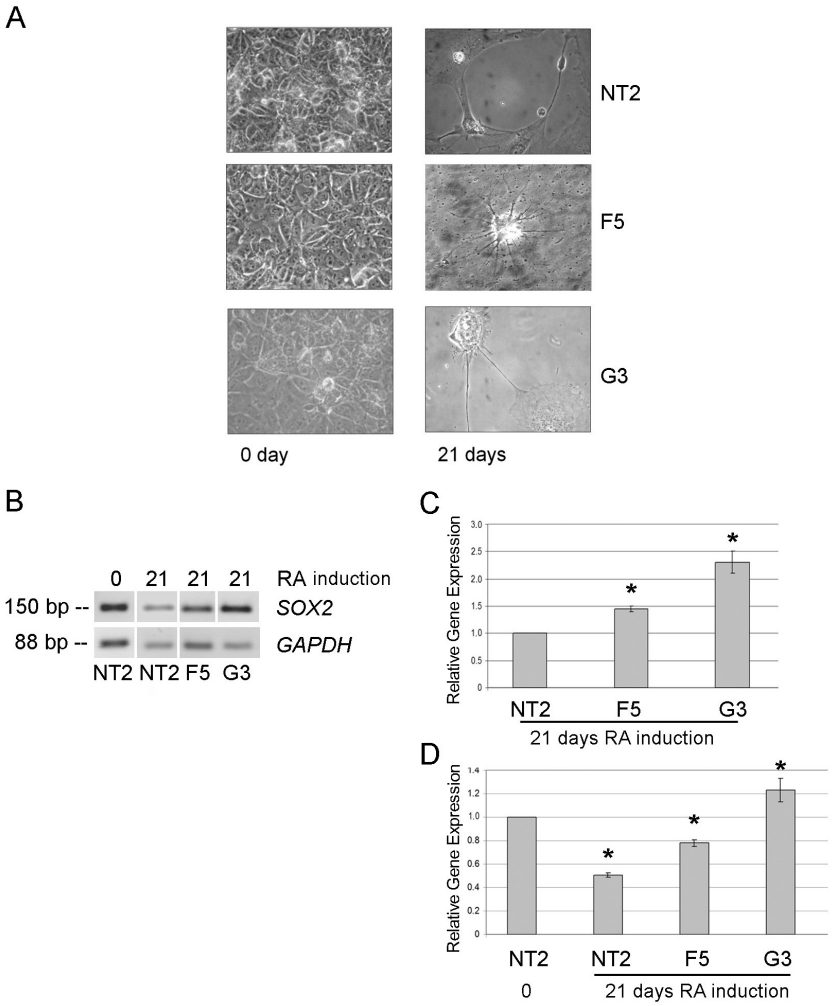


Figure 5. Retinoic acid (RA) induction of NT2/D1 cell clones. **A.** Phase contrast images showing undifferentiated NT2/D1, F5 and G3 cell clones (left panels) and their differentiated derivatives after 21 days of treatment with RA (right panels). **B.** Semi-quantitative RT-PCR analysis of overall *SOX2* expression 21 days after RA induction. F5 and G3 cell clones retained significant *SOX2* overexpression (by approximately 1.4- and 2.3-fold, respectively) compared to NT2/D1 cells. *SOX2* expression in NT2/D1 cells was downregulated by 2-fold when compared with their uninduced counterpart cells. Three independent experiments were performed and representative composite images are shown, with white vertical lines separating merged gel fields. Bands were digitalized, quantified with the ImageJ software and normalized for GAPDH values. The relative *SOX2* gene expression is calculated as a fold expression compared to NT2/D1 cells treated with RA for 21 days (**C**) or compared to not treated NT2/D1 cells (**D**), which were set as 1. Data of three independent experiments are presented in a histogram as the means ± SD. NT2 = NT2/D1 cells; F5, G3 = NT2/D1 cell clones; 0 = no RA induction. *P < 0.05.

Expression of neural markers known to be involved in the formation of the vertebrate nervous system, such as *neuroD1* and synaptophysin, was examined after 21 days of induction by RA (Figure 6). The expression of *neuroD1* was not detected in uninduced cell clones and the parental cell line (Figure 6A). Relative quantification of *neuroD1* expression revealed that expression of this neural marker in F5 and G3 cell clones treated with RA for 21 days is approximately 3.9- and 5.3-fold higher, respectively, when compared to induced NT2/D1 cells (Figure 6B). On the other hand, expression of synaptophysin was observed in uninduced cell clones and the parental cell line (Figure 6A,C). After 21 days of induction with RA, synaptophysin expression was increased in all cells analyzed. In parental cell line expression this increase is approximately 3-fold, while in F5 and G3 cell clones expression this increase is approximately 6.3- and 10-fold, respectively, when compared to uninduced parental cells. Interestingly, expression of this marker in induced F5 and G3 cell clones is approximately 2- and 3.2-fold higher, respectively, when compared to induced parental cells.

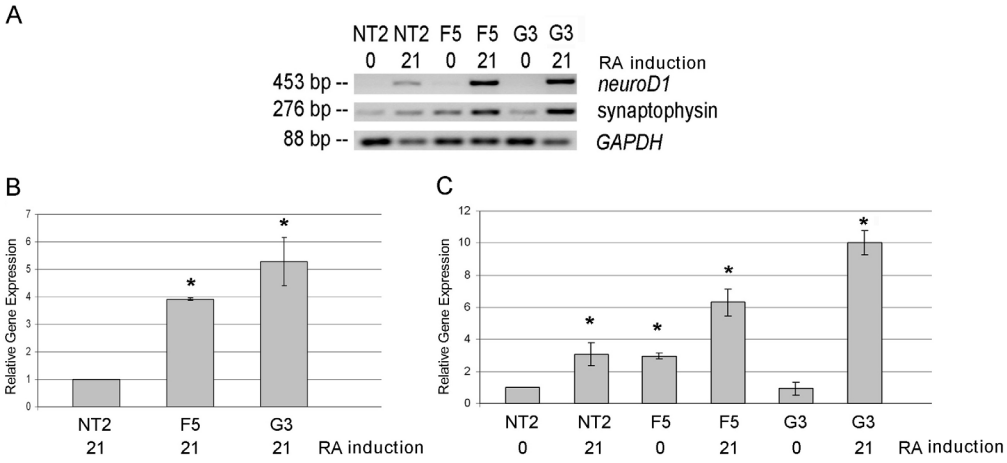


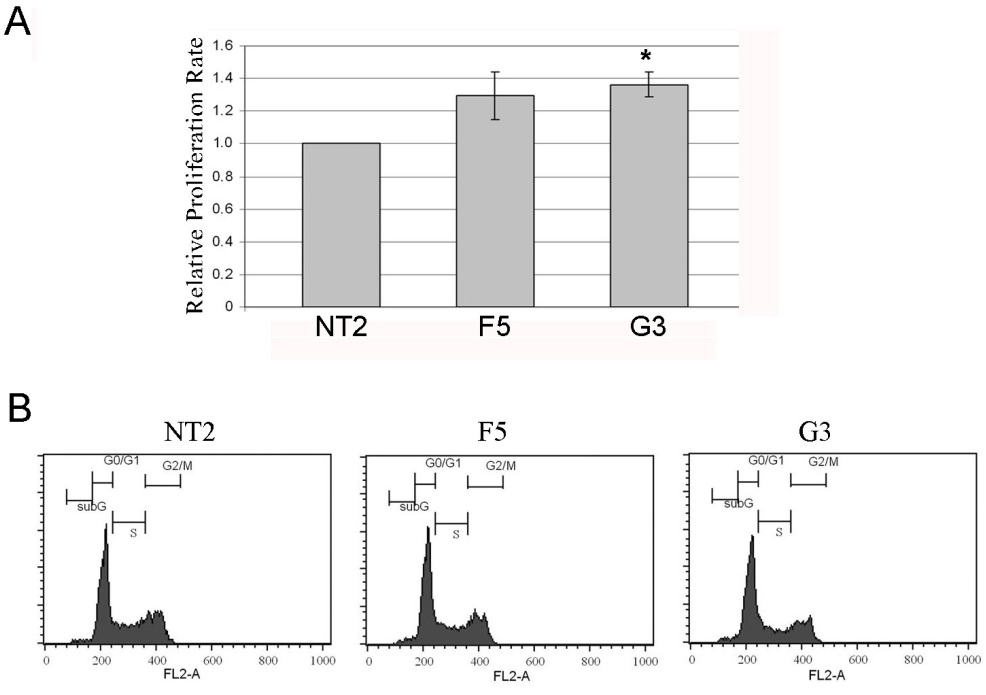
Figure 6. Semi-quantitative RT-PCR analysis of *neuroD1* and synaptophysin expression in NT2/D1 cell clones after 21 days of retinoic acid (RA) treatment. **A. B.** RT-PCR analysis of *neuroD1* in cell clones and parental NT2/D1 cells. **A. C.** RT-PCR analysis of synaptophysin in cell clones and parental NT2/D1 cells. Expression of *neuroD1* and synaptophysin is increased in induced cell clones compared to induced parental cells. Three independent experiments were performed and a representative image is shown. Bands were digitalized, quantified with the ImageJ software and normalized for GAPDH values. The relative *neuroD1* (B) or synaptophysin gene (C) expression is calculated as a fold expression compared to NT2/D1 cells treated with RA for 21 days (B) or compared to not treated NT2/D1 cells (C), which were set as 1. Data of three independent experiments are presented in a histogram as the means ± SD. NT2 = NT2/D1 cells; F5, G3 = NT2/D1 cell clones; 0 = no RA induction. 21 = 21 days of RA treatment. *$P < 0.05$.

Taken together, up-regulation of *neuroD1* and synaptophysin expression implies that *SOX2*-overexpressing cell clones are able to enter in RA-dependent pathway of neural differentiation, even in the presence of elevated *SOX2* expression.

Proliferation rate of *SOX2*-overexpressing NT2/D1 cell clones

Previous studies demonstrated that *SOX2* up-regulation could promote (Lang et al., 2011; Tompkins et al., 2011) or inhibit cell proliferation (Otsubo et al., 2008). Accordingly, we

tested whether *SOX2* overexpression has an influence on cell proliferation of NT2/D1 cells. By performing an MTT cell proliferation assay, we detected that the growth rate of the G3 cell clone is approximately 1.3-fold higher when compared to parental cells (Figure 7A). Since it was previously reported that the altered expression of *SOX2* can influence cell cycle transition (Boyer et al., 2005; Chen et al., 2008; Otsubo et al., 2008), we performed flow cytometry analysis by PI nuclei staining (Figure 7B). This analysis revealed that the relative distribution of cells in cell cycle phases was not altered in F5 and G3 cell clones when compared to parental NT2/D1 cells. Moreover, this analysis demonstrated that neither cell clone had an increased population of cells undergoing apoptosis (subG phase, Figure 7) as previously reported (Mitsui et al., 2003).



	subG	G0/G1	S	G2/M
NT2	1.18±0.13	50.09±2.13	24.72±1.07	24.63±1.93
F5	1.99±0.39	52.53±0.44	24.01±2.09	21.97±1.63
G3	2.08±0.79	52.86±5.18	22.47±2.06	23.11±5.31

Figure 7. Proliferation and cell cycle analysis of NT2/D1 cell clones. **A.** MTT cell proliferation assay. The growth rate of G3 cell clone was significantly changed when compared to parental NT2/D1 cells. Data are means ± SEM (N = 12). **B.** Flow cytometry by propidium iodide nuclei staining. The relative distribution of cells in cell cycle phases was unaltered in F5 and G3 cell clones when compared to parental NT2/D1 cells. No significant population of cells undergoing apoptosis (subG phase) was observed. Three independent experiments were performed and representative ones are shown. Table = mean values of cell fractions in subG, G0/G1, S, and G2/M ± SD (N = 3). *P < 0.001.

DISCUSSION

Sox2, a universal marker of pluripotent stem cells, is a transcription factor that plays a number of significant roles during embryonic development in vertebrates (Papanayotou et al., 2008). This gene is expressed at high levels in neuroepithelial stem cells and its expression persists in neural stem/progenitor cells throughout adulthood (Miyagi et al., 2006). Sox2 is highly expressed in ES and EC cells (Perrett et al., 2008; Schoenhals et al., 2009) and several studies have focused on modulating its expression in these cells in order to elucidate the role of this transcription factor in stem cell pluripotency and self-renewal (Zhao et al., 2004; Boer et al., 2007; Masui et al., 2007; Kopp et al., 2008; Adachi et al., 2010).

Although the role of the *Sox2* gene is extensively studied in ES cells, little is know about its function in the neural differentiation of EC cells. Accordingly, the aim of this study has been to generate *SOX2*-overexpressing NT2/D1 cell clones in order to elucidate the role of this transcription factor in NT2/D1 cell pluripotency, proliferation and differentiation. The study was initiated by engineering NT2/D1 cells overexpressing the human *SOX2* gene. In order to avoid massive cell death, observed when Sox2 levels were elevated at least 4-fold above the endogenous level (Mitsui et al., 2003; Kopp et al., 2008; Tonge and Andrews, 2010), we generated cell clones with *SOX2* expression driven by cytomegalovirus immediate-early promoter (CMV-IE), which exhibited weak activity in ES and EC cells (Niwa et al., 1991; Liew et al., 2007). The use of CMV-driven constitutive overexpression in NT2/D1 cells has been reported for several genes, such as pluripotency-related fibroblast growth factor-4 (FGF-4) and p27, as well as for FLJ11259/DRAM (Maerz et al., 1998; Baldassarre et al., 2000; Kerley-Hamilton et al., 2007). Using this expression system we obtained 54 resistant colonies. However, although many colonies were produced, we established only 16 cell clones, presumably since *SOX2* overexpression induced extensive cell death, as previously reported (Mitsui et al., 2003; Kopp et al., 2008; Tonge and Andrews, 2010). Southern blot confirmed integration of the construct in three cell clones, while semi-quantitative RT-PCR revealed F5 and G3 cell clones with *SOX2* expression 1.7- and 3.1-fold above the endogenous level, respectively. The increase in *SOX2* expression obtained, although not very high, is in line with *SOX2* overexpression achieved in previous studies showing that Sox2 expression of approximately 2-4-fold above the endogenous level affects expression of pluripotency-related genes and triggers differentiation (Kopp et al., 2008; Adachi et al., 2010). Additionally, it has been reported that the CMV driven-expression system is active not only in undifferentiated ES cells, but also in their differentiated derivatives (Bagchi et al., 2006). In line with these data, we have shown that *SOX2* overexpression is active, not only in uninduced NT2/D1 cell clones, but also after treatment with RA for 21 days.

Both generated *SOX2*-overexpressing NT2/D1 cell clones exhibited morphology indistinguishable from that of parental NT2/D1 cells. Similarly, transient Sox2 overexpression of approximately 3-fold above endogenous level in F9 EC cells did not reveal any phenotypic effects (Boer et al., 2007). Also, it has been shown that forced expression of Sox2 does not alter the undifferentiated, self-renewing phenotype of ES cells (Zhao et al., 2004).

The transcription factors Sox2, Oct4 and Nanog are recognized as master regulators that orchestrate mammalian embryogenesis as well as the self-renewal and pluripotency of ES cells (Rizzino, 2008). They play a key role in determining the fate of ES cells, regulating two distinct and opposing functions: self-renewal and differentiation (Rizzino, 2008). Besides di-

recting the expression of target genes, *Sox2*, *Oct3/4* and *Nanog* regulate their own expression *via* positive-feedback loops (Boyer et al., 2005). In both *SOX2*-overexpressing NT2/D1 cell clones analyzed in this study, *OCT4* expression was down-regulated. Detected down-regulation is in concordance with results obtained in ES and EC cells overexpressing *SOX2* (Boer et al., 2007; Adachi et al., 2010). Interestingly, we have observed opposite effects of *SOX2* overexpression on *NANOG* in two analyzed cell clones, with the up-regulation of *NANOG* in G3 and down-regulation in the F5 cell clone (Figure 4C). The role of Nanog in the interplay of key pluripotency factors is still poorly understood. Recent reports have postulated that maintenance of pluripotency does not require the constant presence of Nanog and that Nanog levels vary and reversibly change under appropriate self-renewal conditions (Chambers et al., 2007; Glauche et al., 2010). Taken together, we might postulate that observed differences in *NANOG* expression in the NT2/D1 cell clones analyzed are not directly dependent on *SOX2* levels. Alternatively, it is possible that *SOX2* affects *NANOG* differently at different expression levels.

Previous studies demonstrated that up-regulation of *Sox2* expression is associated with increased cell proliferation in the auditory nerve after injury (Lang et al., 2011), as well as in respiratory epithelial cells (Tompkins et al., 2011). On the other hand, SOX2 overexpression inhibited cell proliferation through cell-cycle (G1) arrest and apoptosis in gastric epithelial cell lines (Otsubo et al., 2008). In this study, the G3 cell clone exhibits increased ability to proliferate. Although MTT assay revealed that the growth rate of the G3 cell clone was higher, the relative cell cycle distribution of these cells was not altered, when compared to NT2/D1 cells. We might postulate that mild overexpression of the *SOX2* gene in the G3 cell clone may well correlate with shortening of all four cell cycle phases, with no change in the relative distribution of cells in G1, S, G2, and M phases, as previously described for Gfi-1 overexpressing Jurkat T-cells, which also have an increased proliferation rate (Karsunky et al., 2002). Furthermore, sustained viability of the cell clones analyzed was demonstrated by flow cytometry, which did not reveal a notable population of apoptotic cells.

One of the most important features of NT2/D1 cells is the ability to differentiate towards ectodermal lineage upon RA induction (Andrews, 1984). In response to RA, NT2 cells differentiate into neurons expressing transcription factors typically observed in the neurons found in dorsal and ventral forebrain, hindbrain and spinal cord (Coyle et al., 2011). Importantly, post-mitotic NT2 neurons obtained by treatment with RA are able to generate action potentials and calcium spikes, express, release and respond to neurotransmitters (Houldsworth et al., 2001; Przyborski et al., 2000, 2003; Coyle et al., 2011).

Previously, it was reported that the RA-induced neuronal differentiation of NT2/D1 cells is accompanied by down-regulation of *SOX2* (Stevanovic, 2003). Additionally, it was demonstrated that constitutive expression of *Sox2* in neural progenitors results in maintenance of proliferation and self-renewal, as well as in blocking of neuronal differentiation (Graham et al., 2003). Furthermore, data obtained by Bylund et al. (2003) suggest that the generation of neurons from stem cells depends on the inhibition of Sox1-3 expression by proneural proteins. Accordingly, one of our goals was to analyze the effect of *SOX2* overexpression on neural differentiation.

The three weeks of RA treatment of F5 and G3 cell clones resulted in a small population of cells morphologically resembling neurons, similar to the effect seen in parental NT2/D1 cells (Figure 5). Analysis of *SOX2* expression after 21 days of RA treatment demonstrated that cell clones retained significant *SOX2* overexpression, compared to treated parental cells.

Interestingly, overall *SOX2* expression in the induced G3 cell clone is approximately 1.3-fold higher when compared to uninduced parental NT2/D1 cells (Figure 5) and this cell clone still retains the ability to differentiate.

Differentiation of NT2/D1 cells into neurons could be divided into three sequential phases (Przyborski et al., 2000). Phase 1 is characterized by the accumulation of *nestin* mRNA, while expression of *neuroD1* and synaptophysin is up-regulated during phases 2 and 3, respectively (Przyborski et al., 2000).

In order to analyze whether *SOX2*-overexpressing cell clones are able to enter into RA-dependent neural differentiation, we analyzed expression of *neuroD1* and synaptophysin in cell clones and a parental cell line treated with RA for 21 days. In induced cell clones, as well as the induced parental line, we detected expression of *neuroD1* and synaptophysin. Interestingly, expression of these two markers is higher in induced cell clones, compared to induced parental cells. Presently, we are not able to estimate whether the significant increase in *neuroD1* and synaptophysin expression is the consequence of an increased number of cells expressing those markers. Alternatively, it is possible that *SOX2* overexpression alters the expression of target genes, which in turn, by direct or indirect mechanisms, caused significant up-regulation of *neuroD1* and synaptophysin. Additionally, we might postulate that *SOX2* overexpression could lead to a delay in terminal neural differentiation, which enables intermediate cells to have more time to proliferate before terminally differentiating.

Taken together, we generated two *SOX2*-overexpressing NT2/D1 cell clones having constitutive *SOX2* expression after three weeks of RA treatment. *SOX2* overexpression resulted in altered expression of pluripotency-related genes, altered proliferation rates and altered expression of neural markers. Further study is needed to estimate whether intermediate neural cells demonstrating up-regulation of *neuroD1* and synaptophysin have higher proliferative capacity and whether NT2/D1 cell clones, with constitutive *SOX2* expression, have the ability to form neurosphere and to terminally differentiate.

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