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Minireview

SOX2 gene – master regulator of numerous cellular processes

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Summary. The SOX (Sry-related HMG box) proteins comprise a group of transcription factors that act as key regulators of diverse developmental and physiological processes, ranging from blastocyst and germ layer formation to differentiation into adult tissues and organs. SOX proteins influence survival and proliferation, as well as cell fate decisions and consecutive lineage progression. Accordingly, SOX proteins are involved in multiple events, from maintenance of stem cells pluripotency, to driving their terminal differentiation into specialized cell types. The SOX2 transcription factor is pivotal for early development and the maintenance of undifferentiated embryonic stem cells (ESCs). This transcription factor plays a critical role in directing the differentiation to neural progenitors and in maintaining the properties of neural progenitor stem cells. It is a crucial transcription factor capable of reprogramming differentiated cells and reversing the epigenetic configuration of differentiated cells back to a pluripotent embryonic state. SOX2 has been found to be an immunogenic antigen in several types of cancers, and its overexpression has been reported in several types of solid tumors. Accumulating evidence suggests that SOX2 acts as an oncogene and recent evidence points toward pro-proliferative, prosurvival and/or antidifferentiation roles of the SOX2 protein. Given the crucial role of SOX2 in cell proliferation and/or antidifferentiation and its ability to endow cells with stemness potential, studying the effects of modulation of its expression has additional significance. Accordingly, we manipulated the level of *SOX2* gene expression and generated cell clones that stably overexpress *SOX2*. We have studied the effects of SOX2 overexpression and present some of our recent findings that have highlighted the important roles of SOX2 in the maintenance of pluripotency, proliferation, neural differentiation and in the regulation of the migration capacity of cells. This review also presents our findings related to the interaction and crosstalk between the *SOX2* gene and the Wnt/β-catenin signaling pathway.

Keywords: cell migration, neural differentiation, NT2/D1 cells, SOX2 transcription factor, stemness, Wnt/β-catenin signaling pathway.

Introduction

Sry (Sex-determining Region Y), a founder member of the *Sox* gene family, was discovered in 1990 as a sex-determining gene necessary and sufficient to specify the male phenotype (Sinclair et al. 1990). During the course of *Sry* cloning, a family of related genes was discovered based on their homology to the HMG box of *Sry.* These newly identified genes were named by the acronym *Sox*/*SOX* (in mammals and human, respectively) standing for *Sry*-related HMG box genes.

Further research revealed that SOX proteins constitute a large family of diverse and well-conserved transcription factors present in both vertebrates and invertebrates. At least 30 *Sox* family members are recognized in mammals, and based on HMG box homology and intron-exon structure they have been divided into ten distinct groups designated A-J (Bowles et al. 2000). Based on sequence analysis and functional studies in vertebrates, group B *SOX* genes are further subdivided into subgroup B1 (*SOX1*, *SOX2* and *SOX3*) and subgroup B2 (*SOX14* and *SOX21*), recognized as activators and repressors, respectively (Uchikawa et al. 1999). Nevertheless, accumulating evidence in the past decade suggests that members of SOXB have dual roles in the regulation of target gene expression, acting as either activators or repressors, depending on

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the cellular and genomic context (Liu et al. 2014; Popovic et al. 2014). In addition, functional redundancy was shown among coexpressed members of the same group (Miyagi et al. 2009; Barrionuevo and Scherer 2010).

As a component of the regulatory network, along with other transcription factors, signaling pathways, epigenetic modifiers and microRNAs, SOX proteins govern diverse cellular processes during development, such as maintaining the pluripotency of stem cells, cell proliferation, cell fate decisions/germ layer formation as well as terminal cell differentiation into tissues and organs. Moreover, in adult tissues SOX proteins influence survival, regeneration, cell death and control homeostasis (Reiprich and Wegner 2015; She and Yang 2015).

Like many other developmental regulatory factors, the improper functioning of *SOX* genes, as well as their deregulated crosstalk in crucial signaling pathways, have been implicated in a number of severe clinical disorders and cancers in humans (Lefebvre et al. 2007; Kormish et al. 2010; Castillo and Sanchez-Cespedes 2012).

SOX2 transcription factor

The human *SOX2* gene was discovered and characterized in 1994 (Stevanovic et al. 1994). It is located on chromosome 3q26.3-q27 and encodes for a protein consisting of 317 amino acids (Stevanovic et al. 1994). Studies have revealed that *SOX2*, together with other members of the *SOXB1* family, is expressed in neuroepithelial precursor cells in both embryonic and adult central nervous systems regulating progenitor identity, survival and development (Collignon et al. 1996; Rogers et al. 2013). To date, numerous data revealed its pleiotropic functions in the regulation of various developmental and physiological processes (Fig. 1). Sox2 plays key roles in the maintenance of pluripotency and self-renewal of stem cells, but also in determining stem cell fate and differentiation (Sarkar and Hochedlinger 2013; Zhang and Cui 2014). Additionally, this transcription factor is important for the totipotency of the cells during embryonic preimplantation period (Pan and Schultz 2011) as well as for differentiation of the cells in developing tissues (Sarkar and Hochedlinger 2013; Zhang and Cui 2014). It has been determined that a deficiency of Sox2 causes early postimplantation lethality (Avilion et al. 2003). Furthermore, during adulthood, Sox2 is necessary for proper tissue homeostasis and regeneration (Sarkar and Hochedlinger 2013).

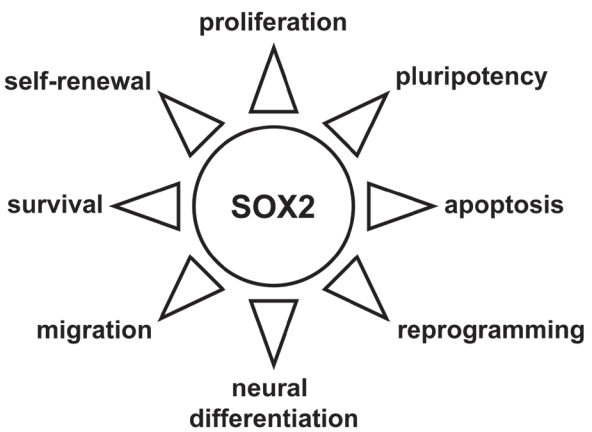

Fig. 1. Schematic presentation of SOX2 involvement in numerous cellular processes (modified from Wang et al. 2016)).

Aberrant expression and mutations of *SOX2* have been identified in several developmental diseases and cancer (Sarkar and Hochedlinger 2013; Weina and Utikal 2014). SOX2 has been established as one of the hallmark participants in various types of cancers (Ren et al. 2016). This is demonstrated through SOX2 involvement in the regulation of various tumorigenesis-related processes, such as cellular proliferation, migration, invasion, metastasis, apoptosis, autophagy, clonal growth, sphere formation and drug resistance (Rizzino 2008; Liu et al. 2013; Wang et al. 2016).

Embryonal carcinoma NT2/D1 cell line as a model system for studying the roles of human SOX2 in different cellular processes

NT2/D1 is a well-characterized human embryonal carcinoma (EC) cell line, derived from a metastasis of a human testicular germ cell tumor. It is a widely characterized pluripotent cell line that resembles embryonic stem (ES) cells in morphology, antigen expression patterns, biochemistry, developmental potential and gene regulation (Andrews 1998). NT2/D1 cells represent malignant counterparts of ES cells and literature data suggest that ES cells resemble cancer stem cells (Ben-Porath et al. 2008). Accordingly, we used NT2/D1 cells as *in vitro* model for studying cancer stem cells (Drakulic et al. 2015).

In the presence of all-trans retinoic acid (ATRA), pluripotent NT2/D1 cells irreversibly differentiate into a network of fully differentiated neurons and astrocytes providing an *in vitro* model for studying human neural differentiation (Przyborski et al. 2000). Our work revealed the dynamic pattern of SOX2 expression during neural differentiation (Stevanovic 2003; Popovic et al. 2014). The high level of *SOX2* expression detected in undifferentiated NT2/D1 cells was transiently downregulated 48 hours after initial exposure to ATRA (Stevanovic 2003). However, the expression was hardly detectable in terminally differentiated neurons and astrocytes (Klajn et al. 2014). These results indicated the importance of fluctuation of SOX2 expression levels during the commitment of stem cells to a differentiated phenotype.

In order to further elucidate the roles of the *SOX2* gene in NT2/D1 pluripotency, proliferation, neural differentiation, migration and adhesion, we have generated stable SOX2 overexpressing NT2/D1 cell clones (Drakulic et al. 2012).

SOX2 and stemness

SOX2, together with OCT4 (octamer-binding transcription factor 4) and NANOG (given as the abbreviation for the mythological Celtic land of the ever-young, "Tir nan Og") (Cavaleri and Scholer 2003), establish the core transcriptional circuit that controls the self-renewal and maintenance of the pluripotency of the stem cells (Rodda et al. 2005). Besides regulating the expression of numerous genes, these three genes control their own expression via positive-feedback loops (Boyer et al. 2005). The fact that SOX2 is a crucial factor for the reversal of somatic cells back to their pluripotent state (Takahashi and Yamanaka 2016) demonstrates its pivotal role in the maintenance of cell pluripotency. Considering that SOX2 is part of an integrated and self-controlling network, the appropriate level of its expression is critical to sustain the stemness phenotype. Accordingly, literature data revealed that SOX2 overexpression reduced the level of OCT4 and NANOG in human embryonic stem cells (Adachi et al. 2010). In agreement with these observations, we detected downregulation of *OCT4* gene expression in NT2/D1 cells with constitutive SOX2 overexpression at the mRNA (Drakulic et al. 2012) and protein levels (Fig. 2A). In accordance with literature data, our results demonstrate that the transition from proliferation to differentiation is accompanied by complete OCT4 downregulation. The same effect was seen in NT2/D1 cells with constitutive SOX2 overexpression (Fig. 2A). However, even though SOX2-overexpressing NT2/D1 cells retain significant SOX2 overexpression after 21 days of ATRA treatment as shown at both the mRNA (Drakulic et al. 2012) and protein levels (Fig. 2B), they maintain the ability to differentiate (Drakulic et al. 2012; Klajn et al. 2014).

Many reports reveal the important role of SOX2 in regulation of the proliferation of numerous progenitor cells (Sarkar and Hochedlinger 2013). Although our results indicate that SOX2 overexpression stimulates proliferation in embryonal carcinoma stem cells (Drakulic et al. 2012), there are numerous publications implying that SOX2 overexpression could either promote (Sarkar and Hochedlinger 2013) or inhibit cell proliferation (Otsubo et al. 2008) depending

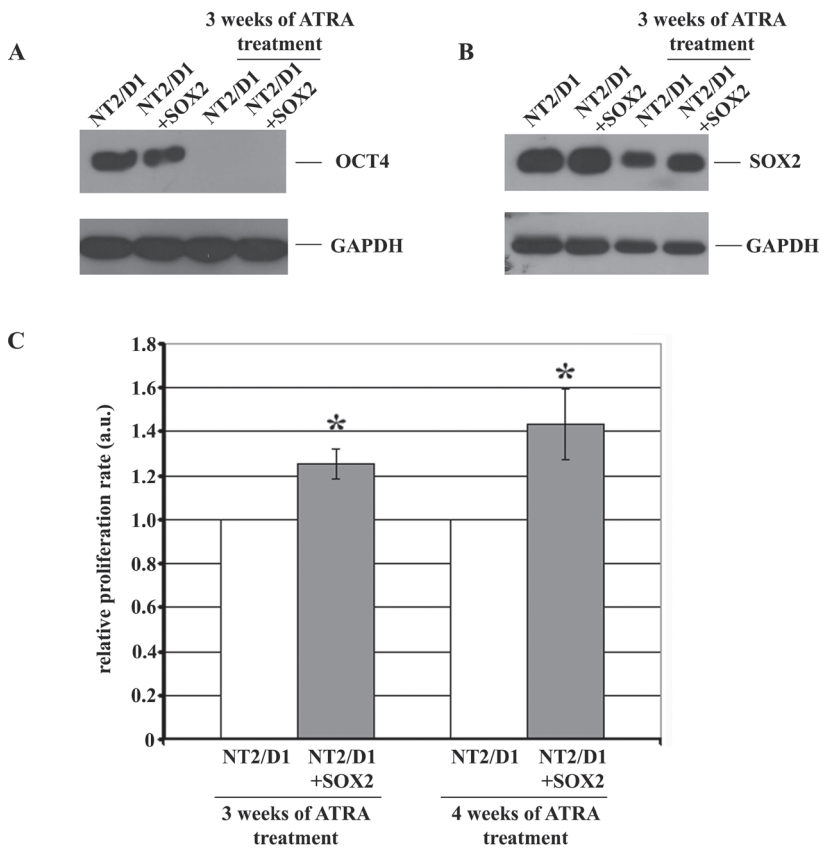

Fig. 2. Western blot analyses of OCT4 (**A**) and overall SOX2 expression (**B**) in parental NT2/D1 cells and NT2/D1 cells with constitutive SOX2 overexpression (NT2/D1+SOX2), both in the undifferentiated state and after three weeks of ATRA-induced neural differentiation. Analysis of the expression of stem cell markers (OCT4 and SOX2) were carried out on the total cell lysates. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) served as a control of equal protein loading. (**C**) Analysis of the effect of SOX2 overexpression on NT2/D1 cell proliferation rate during ATRA-induced neural differentiation. The measurements were performed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. The relative proliferation rate is expressed as the fold increase with respect to parental NT2/D1 cells (set as 1). Data from four independent experiments are presented in the histogram as the means±SEM. *P < 0.05.

on the cellular context. Moreover, our results suggest that SOX2 overexpression stimulates cell proliferation during the whole course of ATRA-induced neural differentiation (Fig. 2C).

SOX2 and neural differentiation

The expression of the SOX2 transcription factor together with its SOXB1 counterparts in vertebrates is associated with the acquisition of neural progenitor identity (Pevny and Placzek 2005) and the maintenance of progenitor population in an undifferentiated state in both developing and adult nervous systems (Bylund et al. 2003; Pevny and Placzek 2005). The effect of SOX2 on neural differentiation is highly dependent on its expression level. While forced SOX2 expression inhibits neuronal differentiation of neural progenitor cells, SOX2 downregulation leads to premature cell-cycle exit and initiation of neuronal differentiation (Bylund et al. 2003; Graham et al. 2003; Stevanovic 2009). In line with these data, our results demonstrate that constitutive SOX2 overexpression interferes with the process of neural differentiation and its final outcome (Klajn et al. 2014). Stable SOX2 overexpression in NT2/D1 cells reduced the number of mature MAP2 (Microtubule-Associated Protein 2) positive neurons, while no difference in the number of GFAP (Glial Fibrillary Acidic Protein) positive astrocytes was detected. In-depth analysis at the single-cell level showed that SOX2 downregulation correlated with the acquisition of both neuronal and glial phenotypes. Interestingly, while SOX2 was completely downregulated in mature neurons, astrocytes with a low level of SOX2 expression were detected (Klajn et al. 2014). It is intriguing that despite its suppressive effect on neurogenesis, SOX2 binds to regulatory regions of neural genes in neural progenitor cells and keeps these genes in a poised state. Therefore, SOX2 predetermines cells for neural differentiation and, at the same time, inhibits their precocious maturation (Bergsland et al. 2011; Wegner 2011). In addition, it was shown that mutations in *SOX2* cause neurodegeneration and impaired adult neurogenesis (Ragge et al. 2005). These data collectively suggest that important stages of neurogenesis, such as the delicate balance between stemness and neural differentiation, as well as lineage-specification and the ratios of neuronal and glial cell types, depend on the SOX2 expression level. These define the SOX2 transcription factor as one of the key elements necessary for proper neural development.

SOX2 interaction with Wnt signaling pathway

In the last decade, *SOX* genes have emerged as important players in various signaling pathways, both during development and in numerous human diseases, including cancer (Kormish et al. 2010). Early vertebrate embryogenesis and the balance between stemness and the differentiation of ES cells are regulated by numerous factors, amongst which the interaction of the SOX2 protein and Wnt/β-catenin signaling represents a key mechanism. In our previous work, we have analyzed the crosstalk between Wnt (Wingless-type Mouse Mammary Tumor Virus integration site) signaling pathway (Nusse et al. 1991) and *SOX2* in NT2/D1 cells (Mojsin et al. 2015). We demonstrated that LiCl-induced activation of Wnt signaling leads to an increased expression of SOX2. We have shown that this transcriptional activation is mediated through the nuclear translocation of β-catenin. Furthermore, we have examined the effects of both transient and constitutive SOX2 overexpression on Wnt signaling activity and detected reduced β-catenin levels in these experimental settings. We demonstrated that this negative feedback loop is the result of GSK3β (Glycogen Synthase Kinase 3β) upregulation and increase in β-catenin levels (Mojsin et al. 2015).

The complexity of SOX2/Wnt interactions is further underlined by reports suggesting that SOX2 can either antagonize or synergize Wnt/β-catenin activity (Kormish et al. 2010). In breast cancer cells, SOX2 binds β-catenin and promotes the transcription of Wnt target genes (Chen et al. 2008), while in gastric cancer, overexpression of SOX2 represses *Cyclin-D1* expression and cell proliferation (Otsubo et al. 2008). We have used quercetin, a bioflavonoid with Wnt-inhibiting activity, in order to elucidate the interplay between SOX2 and the Wnt signaling cascade in NT2/D1 cells. We demonstrated that the inhibition of Wnt signaling decreased the expression levels of stemness factors SOX2, OCT4 and NANOG. At the same time, we detected the promotion of apoptosis and reduction of proliferation, adhesion and migratory potential of NT2/D1 cells. The observed inhibition of pluripotency is a potential mechanism governing the antitumor and antimetastatic effects of quercetin in NT2/D1 cells (Mojsin et al. 2014).

The growing data underline the existence of a finely tuned network between Wnt signaling and SOX2, with further implications in the generation of new therapeutic approaches in cancers and neurodevelopmental disorders with deregulated SOX2/Wnt activity.

SOX2 and cancer

Accumulating evidence suggests that SOX2 acts as an oncogene in numerous cancers, and recent evidence points toward pro-proliferative, prosurvival and/or antidifferentiation roles of this protein (Bass et al. 2009; Sarkar and Hochedlinger 2013). Moreover, there are data indicating a positive correlation between SOX2 expression and an increased capacity of cancer cells for invasion, as documented in tumors of neural/neural crest origin and melanoma (Ikushima et al. 2009; Laga et al. 2010), as well as anchorage-independent growth (Chen et al. 2008). The high level of SOX2 expression in various cancers is associated with poorer prognosis and clinical outcome (Cox et al. 2012). Addition-

ally, a high level of SOX2 expression is one of the molecular characteristics of cancer stem cells (Castillo and Sanchez-Cespedes 2012), which are responsible for cancer occurrence, development, progression, metastasis, high recurrence rate and drug resistance (Weina and Utikal 2014). Taking into consideration that the migration and adhesion of cancer cells are important steps in the metastatic process, we investigated the effect of constitutive SOX2 overexpression on the migration and adhesion capacity of NT2/D1 cells. We detected that increased SOX2 expression changed the speed, mode and path of cell migration, but not the adhesion capacity of NT2/D1 cells. In particular, our results imply that SOX2 overexpression causes faster migration and a switch from cohesive to single-cell motility as demonstrated by time-lapse microscopy (Drakulic et al. 2015) and immunohistochemistry (Fig. 3). Also, under constitutive SOX2 overexpression NT2/D1 cells change their movement from linear to chaotic (Drakulic et al. 2015).

Although SOX2 acts as an oncogene in most type of cancers, the data obtained in gastric cancer indicate that an altered expression of this transcription factor might be associated with its tumor-suppressor role through the inhibition of cell proliferation (Otsubo et al. 2008). These findings further underscore the significance of the regulation of SOX2 expression during cancerogenesis and suggest that, depending on cancer cell type and cellular context, up- or downregulation of SOX2 expression could promote or inhibit malignant properties of cells.

Conclusion

The data presented here point to the *SOX2* gene as a master regulator of numerous cellular processes. Further work is needed to clarify the molecular mechanisms by which *SOX2* exerts its role in the regulation of various cellular processes, and to evaluate its potential as a novel therapeutic target and/or prognostic marker.

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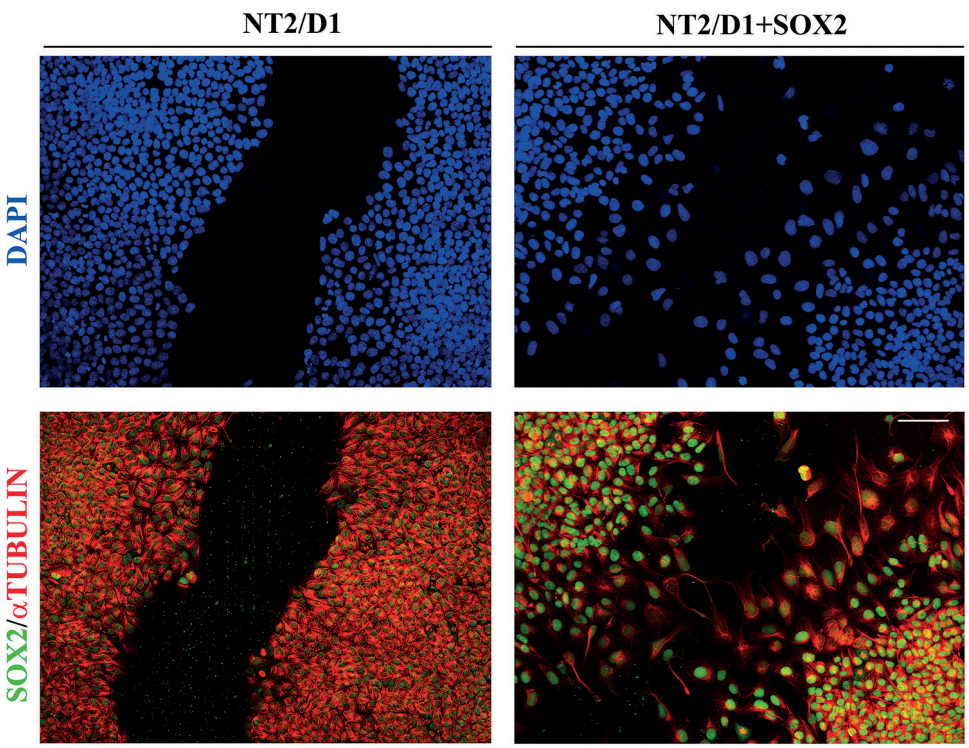

Fig. 3. Effect of SOX2 overexpression on NT2/D1 cell migration analyzed by immunocytochemistry. Parental NT2/D1 cells and NT2/D1 cells with constitutive SOX2 overexpression (NT2/D1+SOX2) were labeled by dual immunofluorescence using the antibody against cytoskeletal protein α-tubulin (red signal on merged images) and anti-SOX2 antibody (green signal on merged images). Nuclei were stained with DAPI (blue signal). Images were taken using a Leica TCS SP8 confocal microscope and applying Leica Microsystems LAS AF-TCS SP8 software (Leica Microsystems). Scale bar: 100 μm.

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