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Superoxide dismutase 1 expression is modulated by the core pluripotency transcription factors Oct4, Sox2 and Nanog in embryonic stem cells

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ABSTRACT

Redox homeostasis is vital for cellular functions and to prevent the detrimental consequences of oxidative stress. Pluripotent stem cells (PSCs) have an enhanced antioxidant system which supports the preservation of their genome. Besides, reactive oxygen species (ROS) are proposed to be involved in both self-renewal maintenance and in differentiation in embryonic stem cells (ESCs). Increasing evidence shows that cellular systems related to the oxidative stress defense decline along differentiation of PSCs. Although redox homeostasis has been extensively studied for many years, the knowledge about the transcriptional regulation of the genes involved in these systems is still limited. In this work, we studied Sod1 gene modulation by the PSCs fundamental transcription factors Oct4, Sox2 and Nanog. We found that this gene, which is expressed in mouse ESCs (mESCs), was repressed when they were induced to differentiate. Accordingly, these factors induced Sod1 promoter activity in a trans-activation assay. Finally, Sod1 mRNA levels were reduced when Oct4, Sox2 and Nanog were down-regulated by a shRNA approach in mESCs. Taken together, we found that PSCs' key transcription factors are involved in the modulation of Sod1 gene, suggesting a relationship between the pluripotency core and redox homeostasis in these cells.

1. Introduction

Embryonic stem cells (ESCs) derive from the inner cell mass of the blastocyst and can give rise to all the cell types of the developing embryo, including the germ line. Therefore, changes in their genome could lead to serious consequences, as fetal lethality or congenital disease (Stambrook, 2007). Accordingly, these cells have a lower mutational rate compared to differentiated cells, in part as consequence of a complex network that secures their genomic stability, which includes high fidelity mechanisms and detoxifying activities, effective DNA repair and low levels of oxidative stress (Saretzki et al., 2008). Moreover, ESCs with accumulated mutations undergo apoptosis or differentiation preserving the population genome (Stambrook, 2007).

Under oxidative stress, the levels of reactive oxygen species (ROS) are augmented. These molecules are produced mainly as a by-product of the mitochondrial respiration and can modify or alter the function of

Abbreviations: ESCs, Embryonic stem cells; LIF, Leukemia inhibitory factor; mESCs, mouse ESC; PSCs, Pluripotent stem cells; Pgk1, Phosphoglycerate kinase 1; Gapdh, Glyceraldehyde-3-phosphate dehydrogenase; ROS, Reactive oxygen species; Sod, Superoxide dismutase

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proteins and lipids, and produce DNA damage (Ufer and Wang, 2011). On the other hand, ROS play an important role as second messengers, being involved in several cellular processes such as proliferation, differentiation and apoptosis (Bigarella et al., 2014). For example, high ROS levels are implicated in promoting the differentiation process in embryonic (Schmelter et al., 2006) and hematopoietic (Ito et al., 2006; Jang and Sharkis, 2007) stem cells. Different enzymes and low molecular weight compounds, such as glutathione, are involved in the cellular antioxidant system that preserves redox homeostasis. Although it is well known that ESCs express high levels of antioxidant enzymes and their expression and activity are reduced throughout differentiation (Circu and Aw, 2010; Ufer and Wang, 2011), knowledge about their transcriptional regulation is still limited. In view of their modulation and the importance of this system, we hypothesized that some of the genes involved in the defense against oxidative stress are regulated by pluripotent stem cells' essential transcription factors. In a previous work, we reported that Oct4 and Nanog modulate a member of the superoxide dismutase family, the mitochondrial Manganese Sod (Sod2/Mn-Sod) gene expression. By *in silico* analysis, we also found predicted binding sites for Oct4, Sox2 and Nanog in a region upstream of Sod1 gene, another Sod family member. Moreover, Sod1 expression pattern showed a decreasing tendency when mouse ESCs (mESCs) were induced to differentiate by a hanging drop differentiation protocol (Solari et al., 2015).

Sod1 was the first member of the Sod family to be characterized and it has been found mainly as a cytoplasmic enzyme (Miao and St. Clair, 2010; Zelko et al., 2002). It is a component of the antioxidant system, since it protects the cell from superoxide anion metabolizing it into oxygen and peroxide, which is substrate for peroxidases. Moreover, Sod1 gene deletion causes oxidative damage in cellular components (Huang et al., 1997) and Sod1 knockout mice display numerous pathologies and a reduced life span (Elchuri et al., 2005; Imamura et al., 2006; Muller et al., 2006; Reaume et al., 1996), highlighting its relevance.

In this work, we studied whether Sod1 gene expression is modulated by Oct4, Sox2 and Nanog, essential transcription factors in ESCs. Our results showed that it was repressed throughout mESCs differentiation and when the transcription factors were specifically down-regulated by a shRNA approach. Moreover, Oct4, Sox2 and Nanog induced Sod1 promoter activity, revealing a relationship between the pluripotency core transcription factors and redox homeostasis.

2. Results and discussion

To investigate whether Sod1 gene is modulated by pluripotency transcription factors, we first performed an *in vitro* differentiation protocol. Since leukemia inhibitory factor (LIF) withdrawal from the culture medium suppresses the expression of pluripotency genes (Cherepkova et al., 2016), this is widely used protocol to study general mechanisms involved in leaving behind the pluripotent state and initiate differentiation. We cultured R1 mESCs in standard stem culture medium with LIF, as control condition, and in absence of this cytokine for 4 days, to promote differentiation. As shown in Fig 1A, we observed the expected changes in ESCs morphology as they exited the pluripotent state. While cells cultured under standard conditions presented a high nucleus/cytoplasm ratio and grew as compact and bright colonies, differentiated cells increased their cytoplasm proportion and grew as a monolayer. To evaluate the exit from the undifferentiated state we measured the mRNA levels of the pluripotency markers Oct4, Nanog and Sox2 which were down-regulated as expected. We also verified that the cells underwent differentiation as the early differentiation marker Fgf5, was induced. Then, we analyzed Sod1 expression and found that it was repressed in differentiated cells (Fig 1B). It was reported that Sod1 protein levels were increased upon differentiation in human ESCs, however, they analyzed a later state of differentiation (Cho et al., 2006). Moreover, since human ESCs represent a more differentiated state than their mouse counterparts (Boward et al., 2016; Davidson et al., 2015), different results may be obtained between these cell types.

To further study Sod1 gene modulation we constructed a reporter vector (pSod1-Luc, Fig. 2A) containing a fragment from −1171 to +75 of Sod1 promoter upstream the firefly luciferase gene. This region includes two putative binding sites for Oct4 and one for complexes of PSC's transcription factors, which were revealed by an *in silico* analysis performed with Genomatix MatInspector software. To evaluate the effect of pluripotency transcription factors in this reporter construction, we performed a trans-activation assay in NIH/3T3 mouse embryonic fibroblast cell line, which has no detectable Oct4, Sox2 or Nanog mRNA levels (Solari et al., 2016, 2015). We co-transfected pSod1-Luc with the vectors encoding for each transcription factor. As shown in Fig. 2, we found that Oct4, Sox2 and Nanog induced the expression of the reporter vector. Moreover, we found a synergic effect of these transcription factors on pSod1-Luc when they were co-transfected simultaneously, since less amount of each factor was required to induce the reporter activity. These results point out that these factors induce the activity of the studied Sod1 promoter region.

Finally, we studied the effect of Oct4, Nanog and Sox2 down-regulation on the endogenous Sod1 gene expression in mESCs by a shRNA approach. We transfected R1 ESCs with vectors that encode specific shRNA targeting Nanog (shNanog), Oct4 (shOct4), Sox2 (shSox2), or eGFP (shGFP) as a control. We first confirmed that each transcription factor was down-regulated by their specific shRNA by RT-qPCR, as shown in Fig. 3A. Moreover, Nanog and Sox2 were repressed in ESCs transfected with shOct4, evidencing the well-known regulation between these factors and in accordance to previous reports (Trouillas et al., 2009). Then, we analyzed Sod1 mRNA levels and found that they were reduced in ESCs transfected with each shRNA (Fig. 3B). Interestingly, we observed a major effect in shOct4-transfected ESCs that could be due to the decrease of both Oct4 and its targets, Nanog and Sox2. These results are in concordance with the above observations and led us to conclude that the pluripotency transcription factors Oct4, Nanog and Sox2 positively modulate Sod1 gene expression.

Previous reports showed that Sod1 gene is often constitutively expressed and not usually modulated as other superoxide dismutases (Miao and St. Clair, 2010). Several transcriptional regulatory elements have been found in the proximal promoter regions of Sod genes and putative binding sites for common transcription factors were revealed by bioinformatic analysis (Miao and St. Clair, 2010). Moreover, the interaction of pluripotent stem cells' transcription factors with Sod1 promoter was also revealed by genome wide ChIP-Seq studies (Chen et al., 2008; Ouyang et al., 2009). Nevertheless, to our knowledge there are no previous reports about the effect of pluripotency transcription factors on Sod1 gene regulation.

In this work we found that Oct4, Nanog and Sox2, the transcription factors that govern gene expression in pluripotent stem cells, modulate the cytoplasmic Superoxide dismutase gene. We have previously found that Oct4 and Nanog also induce another Sod gene, the mitochondrial Manganese Superoxide dismutase (Solari et al., 2015). Since these enzymes are important components of the defense system, responsible for cellular superoxide anion scavenging, our findings highlight an emerging role of the pluripotency transcription factors in the maintenance of redox homeostasis in stem cells.

In the pluripotent state, cells maintain an enhanced antioxidant system, not only preventing oxidative stress and its detrimental consequences, but also securing the preservation of their genome. This safeguard is essential, since in the inner cell mass of blastocysts, pluripotent cells originate all cell types of the organism, including the germ line. Thus, mutations in their nuclear or mitochondrial DNA could have severe consequences. High levels of multiple components of antioxidant system ensure low ROS in pluripotent stem cells. Contrary, the oxidative stress defense decline and a ROS increase takes place along differentiation (Armstrong et al., 2010; Cho et al., 2006; Saretzki et al., 2008, 2004; Sart et al., 2015). In this sense, ROS are proposed to be

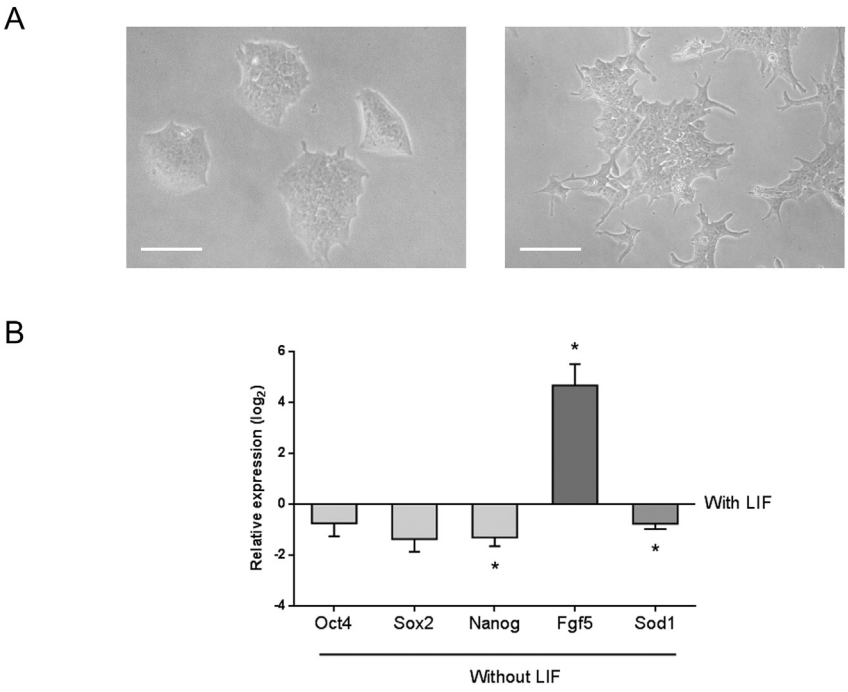

Fig. 1. Sod1 is repressed in ESCs cultured under differentiating conditions. R1 ESCs were cultured for 4 days under standard conditions in the presence of LIF or in the absence of LIF. (A) Representative images of cells cultured with LIF (left panel) and without LIF (right panel) after 4 days of treatment. Scale bars: 100 µm. (B) RNA was extracted and mRNA levels of the indicated genes were measured by RT-qPCR. Gene expression was normalized to the geometrical mean of Gapdh and Pgk1 expression and referred to the control condition (with LIF, baseline). Results are shown as mean ± SEM of at least three independent experiments. Statistical comparisons were performed using randomized block design ANOVA and Tukey Test was used for comparisons between means. Asterisks indicate statistical significance ($p < 0.05$).

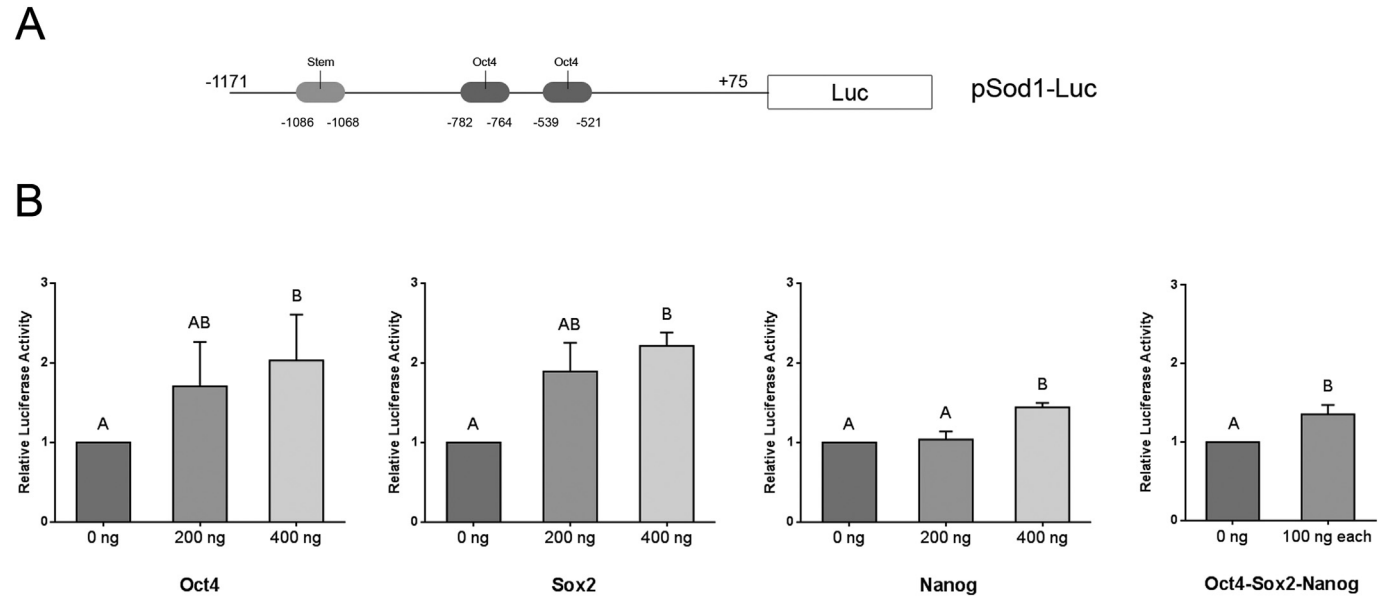

Fig. 2. Oct4, Sox2 and Nanog induce pSod1-Luc reporter. (A) Scheme of pSod1-Luc construction. The putative binding sites for Oct4 and for complexes composed by Oct4, Sox2, Nanog, Tcf3 (Tcf7l1) and/or Sall4b ("Stem") are shown. The numbers below the binding sites indicate the position of each element. (B) NIH/3T3 cells were transfected with pSod1-Luc and the indicated amount of pMXs-Oct4, pMXs-Sox2, pMXs-Nanog, the three vectors simultaneously or none (0 ng, Basal), as indicated. Values were normalized to *Renilla*'s Luciferase activity and referred to the basal condition. Results are shown as mean ± SEM of three independent experiments. Statistical comparisons were performed using randomized block design ANOVA. All treatments were compared using Tukey multiple comparison test, analyzing all possible pairwise simultaneously. Different letters indicate statistically significant differences between treatments ($p < 0.05$).

involved in the balance between self-renewal and differentiation, having an important role as second messengers. It was reported that ROS are involved in osteogenic and adipogenic differentiation of mesenchymal stem cells, and in ESC differentiation towards multiple cell types like cardiomyocytes, neurons, smooth muscle cells and mesendodermal lineage, among others (Nugud et al., 2018; Sart et al., 2015).

In summary, we found that Oct4, Nanog and Sox2 modulate Superoxide dismutase 1 gene transcription. These findings evidence a role of the pluripotency transcription factors in the preservation of redox homeostasis in stem cells, essential for preservation of their genome integrity.

3. Experimental procedures

3.1. Cell culture

R1 ESC line was obtained from ATCC and cultured as previously described (Losino et al., 2013, 2011; Luzzani et al., 2011). Briefly, subconfluent cultures were trypsinized and replated (1:6–1:10 split) every 48–72 h onto 0.1% bovine gelatin (Sigma) -coated tissue culture plates. Cells were propagated in ESC standard medium (high-glucose

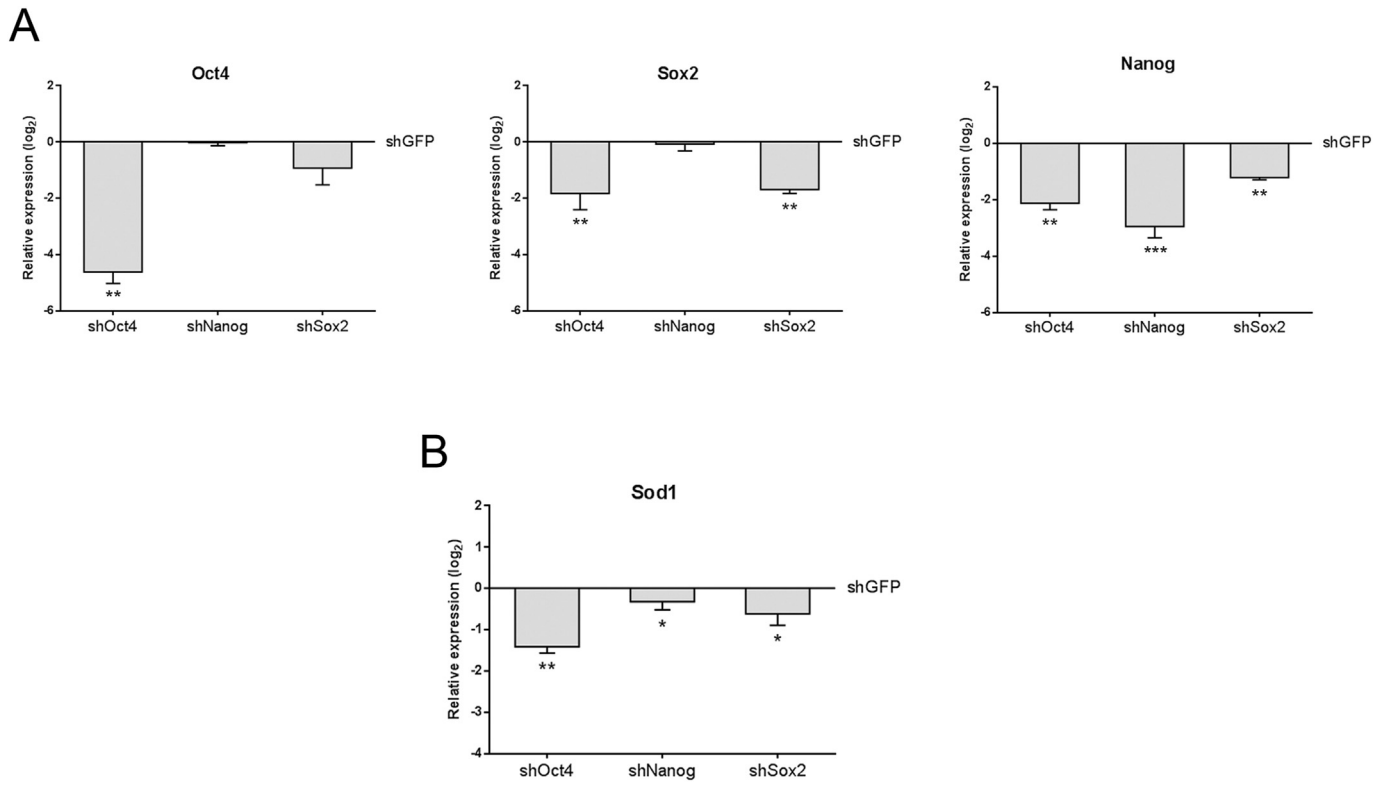

Fig. 3. Sod1 is repressed in ESCs transfected with shRNA targeting Oct4, Sox2 or Nanog. R1 ESCs were transfected with pLKO.1-puro derived vectors targeting eGFP (shGFP, control, baseline), or targeting pluripotent stem cells' transcription factors (shOct4, shSox2 or shNanog), as indicated under each bar. Then, transfected cells were selected with puromycin for 48 hs and RNA was extracted. The mRNA levels of Oct4, Sox2, Nanog (A) or Sod1 (B) were analyzed by RT-qPCR. Gene expression was normalized to the geometrical mean of Gapdh and Pgk1 expression and referred to the control condition. Results are shown as mean ± SEM of at least four independent experiments. Asterisks indicate statistically significant differences between treatments (*$p < 0.05$; **$p < 0.01$; ***$p < 0.005$).

DMEM) (Gibco) supplemented with 15% fetal bovine serum (FBS), 100 mM MEM nonessential amino acids (Gibco), 0.5 mM beta-mercaptoethanol, 2mMl-alanyl-L-glutamine (Gibco), and 100 U/mL penicillin (Gibco) and 100 mg/mL streptomycin (Gibco) with the addition of 1000 U/mL of LIF (Chemicon, ESG1106). For differentiation, cells were cultured in the same standard medium without the addition of LIF for 4 days. For shRNA approach, 500,000 cells were plated in p60 for each condition. NIH/3T3 cell line (ATCC) was cultured in DMEM supplemented with 10% FBS (Internegocios SA), 100 U/mL penicillin (Gibco) and 100 mg/mL streptomycin (Gibco). Subconfluent cultures were replated (1:3–1:5 split) every 48–72 h. For luciferase activity assay, cells were plated onto MW24 plates at a density of 16,000 cells/well.

3.2. Quantitative real time RT-PCR (RT-qPCR)

Total cellular RNA was isolated using TRIzol reagent according to the manufacturer's instructions (Life Technologies). The yield and purity of RNA samples were assessed by the absorbance at 260 nm and 260 nm/280 nm ratio, respectively. One μg of total RNA was retrotranscribed using MMLV reverse transcriptase (Thermo Scientific) and Random Primers (Roche) according to the manufacturer's instructions. Quantitative Real time PCR amplification of DNA was carried out using FastStart SYBR Green Master (Roche) in a Step One Plus Real-Time PCR system (Applied Biosystems, Foster City, California, USA). Specific oligonucleotide sequences are detailed in Refs (Solari et al., 2015; Waisman et al., 2017). A melting curve analysis was performed immediately after amplification at a linear temperature transition rate of 0.2 °C/s from 61 °C to 91 °C with continuous fluorescence acquisition. The amplicon size was confirmed by gel electrophoresis. Raw data were analyzed with LinReg PCR software and N_0 fluorescence values were calculated using the same program. Gene expression was normalized to the geometrical mean of Gapdh and Phosphoglycerate kinase 1 (Pgk1) expression and referred to the control condition as indicated in each case. A no-template blank served as negative control.

3.3. Cloning and construction of reporter vectors

To construct the reporter vector pSod1-Luc, a 1246 bp fragments panning 1171 bp from the promoter region and 75 bp downstream the transcription start site of Sod1 gene was amplified by PCR from R1 ESCs genomic DNA. The oligonucleotides used for amplification and cloning were as follows: Forward 5′ CTAACGCGTGGAGTCCCCTATACCACG CCC 3′; Reverse 5′ CTAGATACCTCGAGAGAGAGAGCAAGACGAGAAG 3′. The amplified fragment was cloned into *Mlu*I and *Xho*I cloning sites in the pGL3-Basic vector (Promega) upstream of the Luciferase gene. Restriction enzymes were obtained from Promega. All constructs were verified by DNA sequencing.

3.4. Transfection and luciferase activity assay

NIH/3T3 cells were co-transfected in 24-well plate with 300 ng of pSod1-Luc reporter and 0, 200 or 400 ng of pMXs-Nanog, pMXs-Sox2, pMXs-Oct4 (Addgene) or 100 ng of the three pMXs vectors simultaneously, as indicated. Transfection was carried out using PEI (Linear Polyethylenimine 25 kDa, Polysciences Inc) with a DNA/PEI ratio of 1:3. For normalization of transfection efficiency, 20 ng of pRL-TK reporter (Promega), constitutively expressing the *Renilla reniformis* luciferase, was included in each transfection assay. After ON incubation, the medium was replaced by fresh medium and 24 h later cells were lysed and assayed for luciferase activity using the Dual Luciferase kit (Promega) on a GloMax Multi Detection System (Promega). Experiments were performed in triplicate and repeated at least three

times.

3.5. Down-regulation of transcription factors by shRNA approach

R1 ESCs plated in p60 were transfected with 3 μg pLKO.1-puro derived vectors expressing specific shRNA (Sigma) targeting Nanog (SHCLND-XM_132755), Oct4 (SHCLND-NM_013633), Sox2 (SHCLND-NM_011443) or eGFP (SHC005), which was used as control vector. Transfection was carried out using PEI with a DNA/PEI ratio of 1:5, and after ON incubation, the medium was replaced by fresh medium. Twenty four hours after transfection, transfected cells were selected for 48 h with puromycin (InvivoGen) at 3 μg/mL final concentration. Then, total RNA was isolated using TRIzol reagent (Life Technologies) and mRNA expression was analyzed by RT-qPCR as described above.

3.6. Statistics and data analysis

Experimental results were presented as mean ± standard error mean (SEM). In Fig. 1 and 2, statistical comparisons were performed using randomized block design ANOVA for biological replicates using Infostat statistical software (Di Rienzo et al., 2014). Tukey Test was used for comparisons between means. For Fig. 3 a linear mixed model was performed in R with Infostat software and DGC Test (Di Rienzo et al., 2002) was used for comparison between means. When necessary, data was transformed with log10. Residuals fitted normal distribution and homogeneity of variance. p values < 0.05 were considered significant.

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