Tumor Biol. (2016) 37:10329–10338
DOI 10.1007/s13277-016-4901-9

ORIGINAL ARTICLE

Overexpression of the non-coding *SOX2OT* variants 4 and 7 in lung tumors suggests an oncogenic role in lung cancer

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Received: 2 December 2015 / Accepted: 20 January 2016 / Published online: 4 February 2016

Abstract Despite the advances in cancer therapy, lung cancer still remains the most leading cause of cancer death worldwide. The long non-coding RNAs (lncRNAs) are recently introduced as novel regulators of human cancers. SOX2 overlapping transcript (*SOX2OT*) is a cancer-associated lncRNA gene that encodes different alternatively spliced transcripts. Here, we investigated the alterations in the preferential expression of different *SOX2OT*s in twenty non-small cell lung cancer (NSCLC) patients by real-time quantitative reverse transcription PCR (qRT-PCR) method. We observed preferential expression of *SOX2OT4* and *SOX2OT7* in lung tumor tissues. The quantitative gene expression analysis revealed that >30 % of NSCLC tumors express *SOX2OT4* (mean = 7.6 times) and *SOX2OT7* (mean = 5.9 times) more than normal tissues, with higher expression in squamous cell carcinoma. Further, we observed overexpression of pluripotency-associated transcription factor, *SOX2* in 47 % of our samples concordant with *SOX2OT* ($R = 0.62$, P value <0.05). Overexpression of *OCT4A* gene was also observed in 36.8 % of tumor tissues. Then, we investigated the effects of *SOX2OT* suppression in lung adenocarcinoma cell line, by means of RNAi. Cell characteristics of colony formation, apoptosis, 2-D mobility, and cell cycle progression were measured in control and treated A549 cells. The *SOX2OT* knockdown significantly reduced the colony formation ability of cancer cells; however, no alterations in the rate of apoptosis were detected. On the other hand, *SOX2OT*-suppressed cells had elevated accumulation in G2/M phase of cell cycle and exhibited limited mobility. Altogether, our findings support a potential oncogenic role for *SOX2OT* in non-small cell lung cancer tumor genesis and *SOX2OT* seems a promising therapeutic candidate for NSCLC.

Keywords Lung cancer · *SOX2 overlapping transcript* · Carcinogenesis · Upregulation

Electronic supplementary material The online version of this article (doi:10.1007/s13277-016-4901-9) contains supplementary material, which is available to authorized users.

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Introduction

Globally, lung cancer is the most common cancer that harbors the highest mortality and morbidity rate among all kinds of cancers worldwide [1–3]. Recent advances in experimental oncology and molecular medicine have provided targeted therapies regarding the genetic alterations in lung cancer. The genetic alterations in *EGFR*, *KRAS*, and *ALK* genes are the best examples of targeted oncogenes in lung cancer therapy [4, 5], whereas the non-protein coding regulators are

underestimated in this context. Most of the human genome (about 98 %) is consisted of non-coding genes that are almost transcribed into ribonucleic acids [6–8]. Subsequently, emerging roles are expected for long non-coding RNAs (lncRNAs) in the diagnosis, prevention, and treatment of lung carcinoma [9–14] like other cancers [15].

SOX2 overlapping transcript (*SOX2OT*) is a long non-coding RNA gene located in chr3q26.33, which is frequently amplified in lung cancer tissues [16, 17]. It encodes different transcript variants with alternative splicing in a tissue-specific manner [18, 19]. However, it cannot encode any protein sequence [20].

Recently, it has been reported that *SOX2OT* is overexpressed in breast, hepatocarcinoma, and esophagus tumor tissues [21–24] and also in lung carcinoma that is associated with poor survival in NSCLC patients [25].

In the present study, first of all, we screened the lung tissue-specific expression of different *SOX2OT*s, and then the expression alterations in adenocarcinoma (AC) and squamous cell carcinoma (SCC) tumors of NSCLC patients ($n=20$) were investigated. It has been reported that *SOX2* and *OCT4*, two of the pluripotency key factors, role as oncogenes in lung tumor genesis and progression [26]. Considering the previously reported association of *SOX2OT* with pluripotency [18, 23], we focused the expression of *SOX2* and *OCT4A* transcription factors synchronously. To study the function of *SOX2OT* in lung cancer, we explored the cellular response of lung adenocarcinoma cell line (A549) to *SOX2OT* suppression in vitro. For this purpose, a series of experiments including colony formation assay, wound closure assay, apoptosis, and cell cycle staining were carried out in SOX2OT:siRNA- and control:siRNA-transfected A549 cells.

Materials and methods

Tissues and sampling

Twenty NSCLC surgical sample pairs (tumor and apparently normal tissues from the same patients) were provided by Tracheal Diseases Research Center, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran. The clinico-pathological information of patients including the age, tumor subtypes, tumor stage and score (according TNM Classification) is provided in Table 1. The research ethics committee of NRITLD and Golestan University of Medical Sciences (code: 92041130) approved the experimental procedure. A signed informed consent form was also obtained from each patient. Small surgical pieces of tumor and non-tumor tissue samples from the same lung lobe of patients were put in a RNase-free Eppendorf tube and immediately snap-frozen in liquid nitrogen, and stored in −80 °C for later RNA extraction.

Cell culture and transfection

Two cell lines of human lung adenocarcinoma cancer cell line, A549 and glioblastoma multiform cell line, U-87 MG were obtained from Pasture institute (Tehran, Iran). Cells were cultured in RPMI1640 medium (Invitrogen, Gaithersburg, MD) supplemented with 10 % fetal bovine serum (FBS; Invitrogen, Gaithersburg, MD), and 100 IU penicillin 100 μg streptomycin per ml (Invitrogen, Gaithersburg, MD), and cultivated in a 98 % humidified and 5 % CO2 incubator.

A general *SOX2OT*-specific siRNA (5′-GGAGAUUGUGACCUGGCUU-3′) capable of hitting both variants 4 and 7 was designed and synthesized by Bioneer Inc (Daejeon, Korea). For siRNA transfection, 5×10^5 cells were seeded in six-well tissue culture plates. After 24 h, the cells (about 80 % confluent) were transfected with scramble (SantaCruse, s-c37007), and SOX2OT (50 or 100 nM) siRNAs, using Lipofectamine 2000 and according to the manufacturers protocol. The efficiency of siRNA delivery was monitored by fluorescent inverted microscope (Nikon, Ti-U model) in cells transfected with FITC-siRNA (Fig. 3a). Forty eight hours later, the cells were harvested for RNA extraction or functional assays.

Colony formation assay

The colony formation potential was measured by routine monitoring of treated cells for 12 days. Briefly, about 200 cells were seeded in a six-well tissue culture plate, before being transfected by siRNAs, 24 h later. Then, transfected cells were incubated in CO_2 incubator and complete medium which were renewed every 3 days. The colonies were washed with PBS and fixed by 4 % paraformaldehyde at room temperature. Crystal violet (0.1 % *W/V*) in PBS was used to stain the colonies for 10 min. Colonies then were washed by PBS and were imaged by digital camera or microscopically. Colonies consisting <50 cells or smaller than 1 mm were excluded from counting. Images were analyzed by ImageJ 1.4 software colony count tool.

Flow cytometry analysis

The siRNA-treated cells were washed with PBS, before being harvested. The annexin V/PI (Sigma-Aldrich) staining was performed for cells according to the manufacturer's instruction. Then, the stained cells were studied by Partec flow cytometry instrument (Partec GmbH, Münster, Germany). The percentage of annexin V and propodium iodide positive cells were measured for early and late apoptosis estimation.

For the cell cycle evaluation, treated cells were washed with PBS and harvested for DNA content staining, 48 h after transfection. Cells were then fixed in cold ethanol (70 %) for 1 h in −20 °C, then were permeabilized with triton X −100 (0.1 %). The RNA content of cells was depleted with 30 min

Table 1 The clinicopathological description of lung cancer patients

Patients (*N*, %)	Age	Histology (*N*, %)	Stage (*N*, %)	Tumor score (*N*, %)
Female (5, 15 %)	60	AC (3, 60 %)	I (1, 20 %)	T1 (1, 20 %)
			II (2, 40 %)	T2 (2, 40 %)
		SCC (2, 40 %)	III (1, 20 %)	T3 (1, 20 %)
			IV (1, 20 %)	T4 (1, 20 %)
Male (15, 85 %)	56.6	AC (11, 73.3 %)	I (3, 20 %)	T1 (2, 13.3 %)
			II (8, 53.3 %)	T2 (7, 46.6 %)
		SCC (4, 26.6 %)	III (3, 20 %)	T3 (4, 26.6 %)
			IV (1, 6.6 %)	T4 (2, 13.3 %)
Total	57.16	AC (14, 70 %)	I (4, 20 %)	T1 (3, 15 %)
			II (10, 50 %)	T2 (9, 45 %)
		SCC (6, 30 %)	III (4, 20 %)	T3 (5, 25 %)
			IV (2, 10 %)	T4 (3, 15 %)

RNaseI (Fermentase) treatment (10 μl/ml) in 37 °C temperature. Then, the cells were stained with Propodium Iodide (10 μg/ml), and the percent of cell population in different phase of cell cycle was analyzed with flow cytometry.

Wound closure assay

The two-dimensional cell migration were measured by percent of gap area in a wounded region. The number of 5×10^5 Cells were seeded in a six-well tissue culture plate and were transfected with siRNA. Twenty four hours later, the culture medium was changed with a 0.5 % serum one to minimize the cell proliferation. The cells were almost confluent at 48 h after transfection when a sterile tip was used to scratch the center of the well. The wounded region was imaged by light microscopy immediately (0 h) and 24 h later. For same fields of images, the wound edges were found and the gap area was calculated by ImageJ v1.4 software. The percentage of wound closure was calculated as (gap area in 24 h/gap area in 0 h) × 100.

Gene expression measurement

The total cellular RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Complementary DNA was synthesized using revert aid first strand cDNA synthesis kit (Thermo Fisher Scientific, Inc) following by DNaseI (Thermo Fisher Scientific, Inc) treatment.

Quantitative measurement of gene expression was carried out by SYBR green real-time PCR kit (Thermo Fisher Scientific, Inc) with specific primers listed in Table 2; for *SOX2* [GeneBank: NM_003106.3], *SOX2OT* [GeneBank: NR_004053.3, NR_075089.1, NR_075090.1, KF981435, and KJ415055], *OCT4A* [GeneBank: NM_002701.4], and *GAPDH* [GeneBank: NM_002046.3].

Thermal cycling was performed in ABI system 7300 (Applied Biosystems, Foster City, CA) scheduled to 95 °C for 10 min (enzyme activation), 40 cycles of 95 °C for 10 s (denaturing), 58 °C for 15 s (annealing), and 72 °C for 30 s (extension). Then, final dissociation curve analysis was performed to ensure the specific PCR product amplification.

The gene expression of tumor tissues was normalized to paired normal tissue of same patient and then the quantitative gene expression fold changes were calculated by the $2^{-\Delta\Delta Ct}$ method, considering *GAPDH* as housekeeping gene.

Statistical analysis

The measurements were carried out in experimental replicates to decrease the artificial error. The SPSS v22 software was used to analyze data statistically. For statistical tests, 95 % confidence interval and P value ≥0.05 were considered. For gene expression results, the mean ± SE of calculated fold changes was compared between SOX2OT:siRNA and Scrambeled:siRNA or between tumor and normal tissues as well as tumor sub-types (squamous and adenocarcinoma) and stages (TNM stages A and B). Student's t test (two tailed) was performed for each gene separately to analyze the tissue gene expression results. The one-way ANOVA and Dunnet's test were used to compare means of measurements in treated cells to control. The correlation coefficient of gene expression was measured by the Spearman's rho test.

Results

Differential expression of *SOX2OT*-spliced variants in lung tumor tissue samples

Using reverse-transcription PCR (RT-PCR), we initially examined the expression pattern of newly identified human *SOX2OT*-spliced variants (transcripts 1–8) in five lung tumor tissue samples, with specific primers for each variant (Table 2). A schematic

Table 2 The specific primer sequences and PCR product length

Primer sequences	Gene	Product (bps)
F1:TCTGTTCAGTATTTGGAAGAAAG;R1:GCAGAGATTATCCGATTTGG	*SOX2OT4*	389
F1; R2: ACATTATTTCTAAGTTGGATATGTC	*SOX2OT5*	398
F1; R3: CATTATTTCTAAGTTGGATTGGA	*SOX2OT6*	111
F1; R4: GCTTGGACCCGCGTG	*SOX2OT7*	104
F2: TTCAGAAACTTATCAAGAGGTTC; R4	*SOX2OT8*	168
F3:TACAGCATGTCCTACTCGCAG;R5:GAGGAAGAGGTAACCACAGGG	*SOX2*	110
F4:TTCGCAAGCCCTCATTTCAC; R6:CGAGAAGGCGAAATCCGAAG	*OCT4A*	308
F5:GAGCGAGATCCCTCCAAAAT; R7:GGCTGTTGTCATACTTCTCATG	*GAPDH*	196

picture of different spliced variants of *SOX2OT* and the location of forward and reverse primers for specific detection of each variant is provided in Fig. 1b. Using theses primers, we examined a potential expression of *SOX2OT* variants in lung tumor surgical specimens. A glioblastoma multiform cell line, U-87 MG, was utilized as a positive control to compare the expression pattern of the variants in two samples. As it is shown in Fig. 1a, no or very low expression was observed for *SOX2OT* transcripts 5, 6, and 8. However, variant 7 showed a moderately high and variant 4 presented a significantly high level of expression in lung tumors. The expression pattern was somehow different from that of U-87 MG glioblastoma cell line which was used as a positive control. The *SOX2OT* variants 4–8 were expressed in U-87 MG cells, with highest expression for variants 4 and 5. The expression of distal *SOX2OT* variants 2 and 3 were not observed in neither NSCLC nor U-87 MG; however, *SOX2OT1* was the only distal

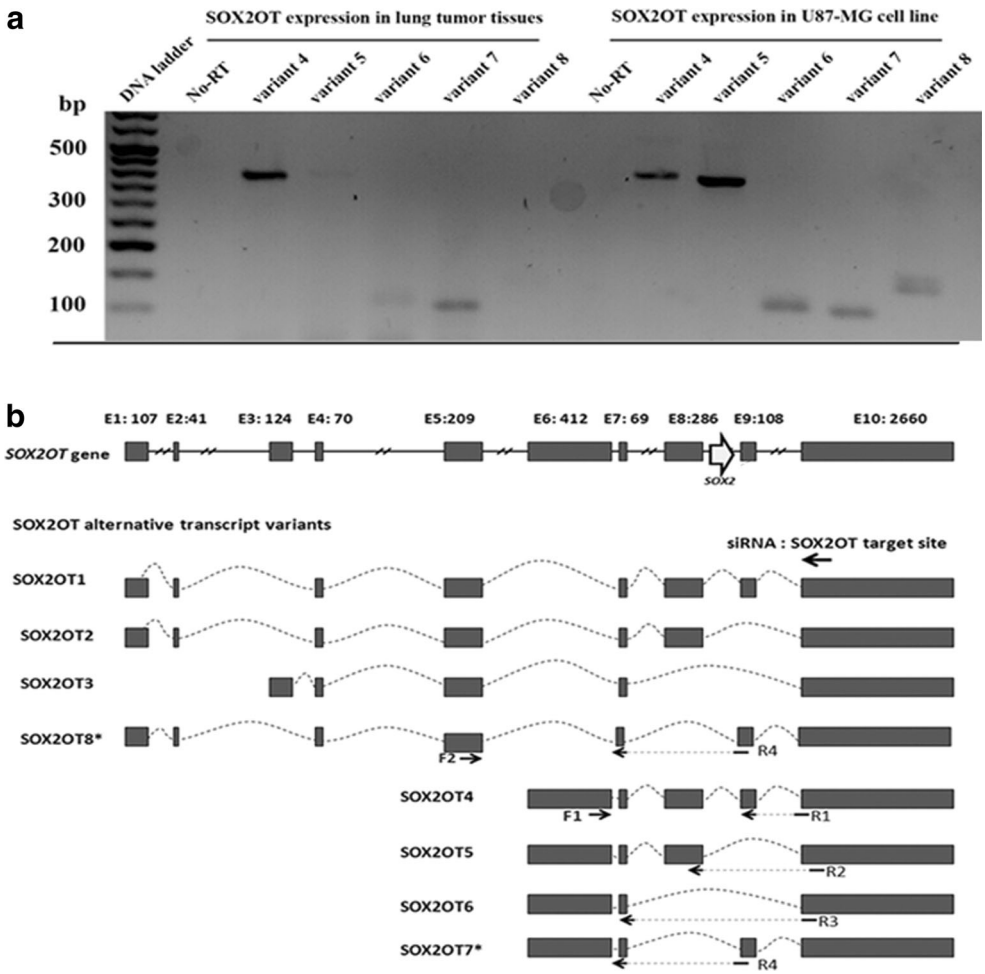

Fig. 1 The specific expression of different *SOX2OT*s (variants 4, 5, 6, 7, and 8) in lung tumor. **a** The expression of *SOX2OT*s in lung tumor was checked by RT-PCR. The U-87 MG glioblastoma cell line was used as positive control and no RT indicates negative control. **b** The schematic presentation of *SOX2OT* gene and its transcripts. The primers and siRNA target sites are indicated by *arrows*. *Asterisk* indicates the non-refseq transcripts

SOX2OT transcript in U-87 MG glioblastoma cell line (data not shown).

Upregulation of *SOX2OT* transcripts 4 and 7 in lung tumor tissues

The relative expression of two most abundantly expressed *SOX2OT* variants (transcripts 4 and 7) were measured in lung tumor tissues of adenocarcinoma and squamous cell carcinoma ($n=20$) by qRT-PCR. The data were normalized to samples from paired apparently normal lung tissues obtained from same patients ($n=20$).

Our data revealed that *SOX2OT* variants 4 and 7 were upregulated around six times in lung tumor tissues, compared to their non-tumor counterparts (Fig. 2a). However, as shown (Fig. 2a and supplementary figure 1), the *SOX2OT* gene expression level was variable in different tumors. A closer look at expression of variants in each sample demonstrated that *SOX2OT7* was upregulated in 36.8 % and *SOX2OT4* in 31.57 % of lung tumor tissues (Table 3). Furthermore, when data was re-examined according to the cancer subtypes, it was noticed that upregulation of *SOX2OT4* is significantly higher in squamous cell lung carcinoma rather than adenocarcinoma subtype. We found higher expression level for *SOX2OT7* in squamous cell carcinoma but the value was not significant (Fig. 2b). However, the overexpression of *SOX2OT* transcripts was not significantly different in different tumor sizes and early vs. metastatic tumors, considering the TNM stages (data not shown).

Concomitant upregulation of *SOX2* with *SOX2OT* in lung tumor tissues

It is assumed that *SOX2OT* regulates pluripotency through controlling *SOX2* expression. In order to investigate a possible association between the expressions of the genes in lung tumor tissues, we explored the expression alteration of *SOX2* and *SOX2OT* genes in tumor vs. non-tumor samples. The expression of *OCT4A* was also examined in each sample as a control. Our data revealed that *SOX2* was upregulated in tumor samples, as the same level of *SOX2OT* variants (in 47 % of the samples, about six folds), with higher expression in squamous cell carcinomas, compared to adenocarcinoma (Fig. 2 and Table 3). We also observed an upregulation for *OCT4A* (in 36.8 % of the samples, more than two-fold increase) in lung tumor samples, compared to apparently normal tissues obtained from the same patients. However, the relative upregulation was not different in cancer subtypes.

The Spearman's rho correlation was calculated for *SOX2*, *SOX2OT* variants 4 and 7, and *OCT4A* expression in our lung tissue samples. As was expected, the expression of *SOX2OT*

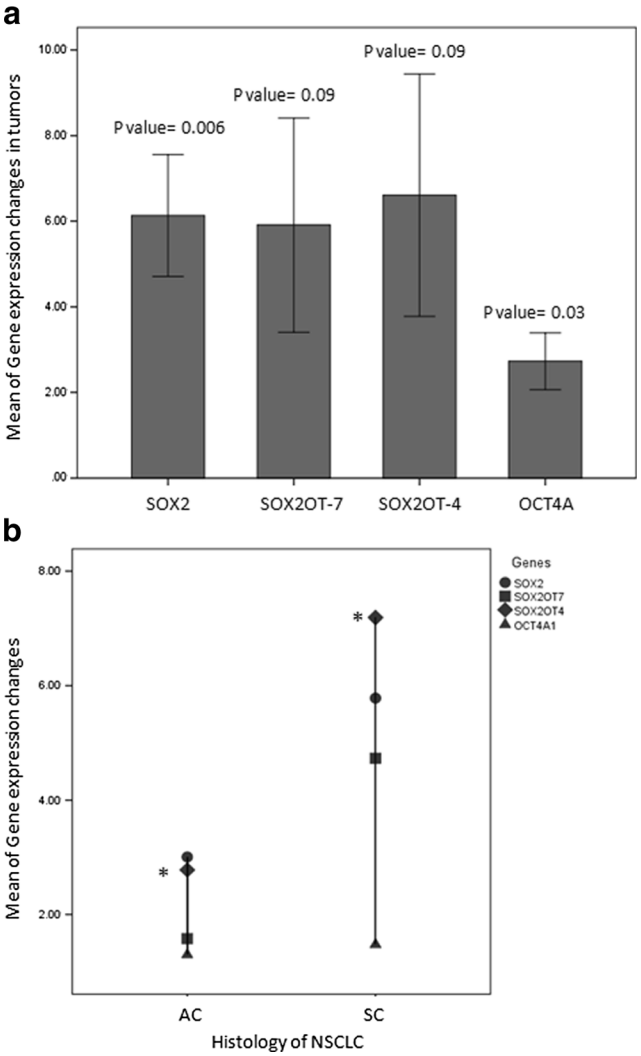

Fig. 2 The relative gene expression of lung tumors normalized to paired normal tissues. **a** The mean of gene expression alteration is presented for *SOX2*, *SOX2OT4*, *SOX2OT7*, and *OCT4A* in tumor tissues. **b** The gene expression changes is visualized in different histology of lung cancer categorized to two adenocarcinoma (*AC*) and squamous cell carcinoma (*SC*) cancer subtypes. The normal tissue expression level is considered as one. The *error bars* indicate ±1SE. **p* value <0.05

variants 7 and 4 ($R=0.8$, P value <0.005) and pluripotency genes *SOX2* and *OCT4A* ($R=0.55$, P value <0.05) were significantly correlated with each other. We also found that the expression of *SOX2* and *SOX2OT7* genes are positively correlated in lung tumor tissues ($R=0.62$, P value <0.05).

SOX2 was significantly downregulated in SOX2OT:siRNA-treated cells

A qRT-PCR approach was utilized to confirm *SOX2OT* suppression in siRNA-transfected A549 cells. To examine a potential regulatory role of *SOX2OT* on pluripotency genes' expressions, the expression levels of *SOX2* and *OCT4A* genes were also determined.

Table 3 The statistics for gene overexpression in lung tumor tissues of different histology

Genes	Histology	Mean	Minimum	Maximum	% samples with overexpression
SOX2	SCC	7.6200000	2.53000	10.85000	15.78
	AC	6.2600000	1.67000	14.80000	31.57
	Total	6.7133333	1.67000	14.80000	47
SOX2OT-7	SCC	11.5000000	3.00000	20.00000	10.52
	AC	3.6760000	1.30000	8.42000	26.31
	Total	5.9114286	1.30000	20.00000	36.8
SOX2OT-4	SCC	10.5150000	1.03000	20.00000	10.52
	AC	6.1725000	1.03000	12.59000	21
	Total	7.6200000	1.03000	20.00000	31.57
OCT4A	SCC	3.5500000	2.64000	4.46000	10.52
	AC	6.1725000	1.03000	12.59000	26.31
	Total	2.9942857	1.07000	6.10000	36.8

As expected, *SOX2OT* expression was downregulated in A549 cells transfected with SOX2OT:siRNA (50 and 100 nM). Interestingly, the expression level of *SOX2* was also declined in *SOX2OT* knockdown cells, in comparison to the cells transfected with scrambled siRNA (Fig. 3b). Our data demonstrated a significant positive correlation between *SOX2OT* and *SOX2* gene expression alterations in A549 ($R = 0.66$, P value $= 0.05$); however, the expression level of *SOX2OT* and *OCT4A* genes was not correlated significantly.

SOX2OT suppression revealed its part in proliferation and colony formation of tumor cells

A potential contribution of *SOX2OT* in proliferation and colony formation of tumor cells were examined in siRNA:SOX2OT-transfected A549 cell line, for a period of 12 days post-transfection. As it is shown in Fig. 4, *SOX2OT* knockdown significantly declined the proliferation rate and colony size of transfected cells. The colony formation ability of A549 cells were dramatically declined following *SOX2OT*

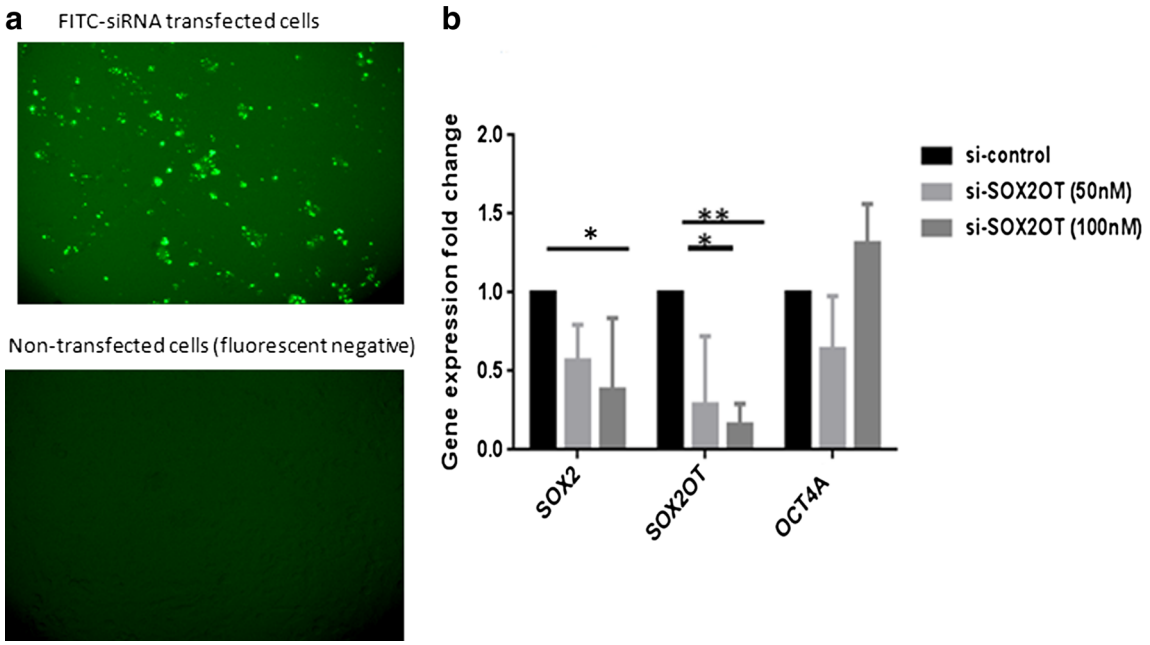

Fig. 3 RNA interference *SOX2OT* knockdown in A549 cell line. **a** The fluorescent microscope image of FITC-siRNA-transfected cells. Green fluorescent signals indicate the Fluorescein-tagged siRNA in transfected cells. The non-transfected cell fluorescent microscope image is shown as a negative control. **b** The expression of *SOX2OT*, *SOX2*, and *OCT4A* were measured concordantly by relative real-time PCR method. The expression of *SOX2* falls upon *SOX2OT* knockdown in SOX2OT:siRNA-transfected cells

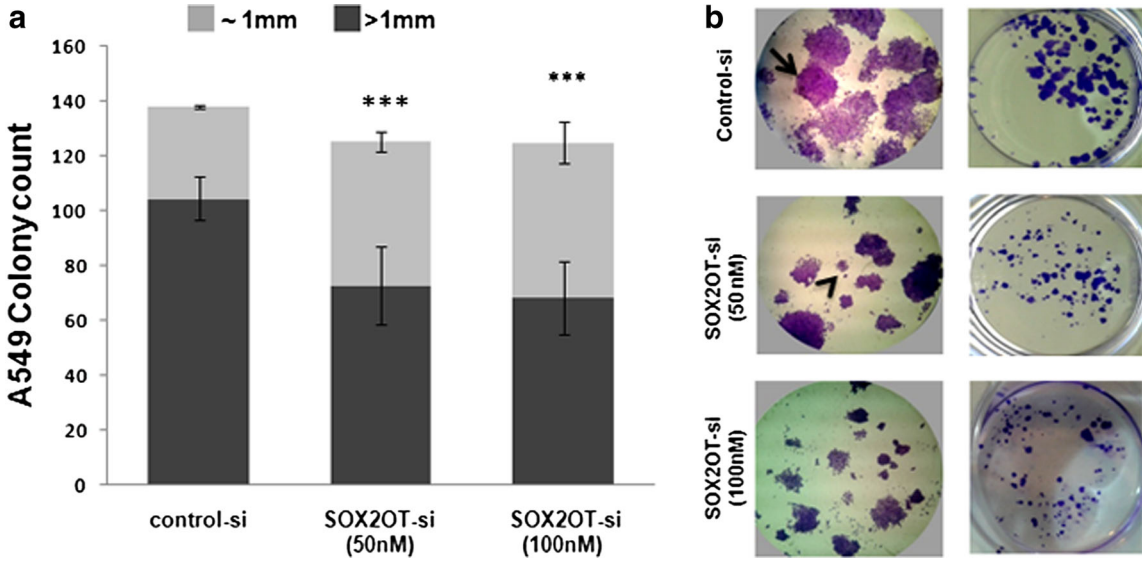

Fig. 4 The colony formation assay of *SOX2OT* knockdown and control cells. The bar chart describes the count of >1- or ~1-mm colonies in SOX2OT or control siRNA-transfected A549 cells (**a**). Crystal violate stained colony images (**b**) are presented macroscopically (*right column*) and microscopically (×40 magnification, *left column*). The *arrow* points to a typical >1-mm colony and *arrow head* shows a ~1-mm one

suppression; however, the overall colony numbers were similar.

SOX2OT has a role in G2/M exit of cell cycle

Cell survival and proliferation are interdependently determining cancer cell growth. *SOX2OT* suppression can decrease cell proliferation either by inhibition of cell proliferation or elevation of death rate, or both. For this reason, the siRNA:SOX2OT-trasfected A549 cells were examined for a possible alteration in the rate of apoptotic cell death. The flow cytometry failed to show any significant changes in the number of annexin V-positive cells, 48 h post-transfection (Fig. 5a).

Since cell death was not there as on behind the declined number and size of colonies in siRNA:SOX2OT-transfected cells, we focused on cell cycle progression of the treated cells as a probable factor behind the anti-proliferation effect of *SOX2OT* suppression. Interestingly, a declined percentage of the cells in G1 phase of cell cycle were observed in *SOX2OT* knocked down A549 cells, which seems to be caused by a blockage of the cells in G2/M phase of cycle (Fig. 5b).

Declined two-dimensional cell migration in A549 cells transfected by SOX2OT:siRNA

Considering the vital importance of cell migration in cancer progression and metastasis, we examined whether the mobility potential of the lung adenocarcinoma cell line A549 is affected by *SOX2OT* knockdown. In this regard, the two-dimensional motility of siRNA-transfected cells was studied with a simple wound closure assay. After 24 h, both concentrations of siRNAs (50 and 100 nM) caused a significant decline in cell mobility of *SOX2OT* knockdown cells, compared to the cells transfected with scrambled siRNA (Fig. 6). However, there was no significant difference in mobility of cells treated with different concentration of siRNA.

Discussion

The lncRNAs which make the majority of human transcriptome [27] intercommunicate with cancer pathogenesis of different tissues [28–30]. A list of lncRNAs expressed differently between normal and NSCLC tumor tissue has been reported, even discriminating squamous cell and adenocarcinoma sub-types [12, 14]. This indicates the importance of lncRNA in molecular pathobiology of lung cancer and also provides new insights in personalized medicine of it.

For example, *SCAL1* elevation is an early event in smokers to protect bronchial airway cells from cigarette smoke side effects, which may enhance the malignancy [31, 32] and two another well-known lncRNAs: *MALAT1* [33–36] and *HOTAIR* [37] are correlated with cancer cell migration and predict the survival and metastasis of NSCLC.

SOX2OT high expression level is associated with different cancer tissues of the esophagus [23], breast [21], and lung [38]. An amplification in 3q26 locus, encompassing *SOX2* and *SOX2OT*, is reported as a common features of squamous cell carcinoma of lung cancer [17].

Previously, Hou Z et al. reported that *SOX2OT* upregulation (>2 folds) in lung tumor tissues (53 %) is associated with poor prognosis. Here, for the first time, we elucidated the preferential expression of different non-coding *SOX2OT*-

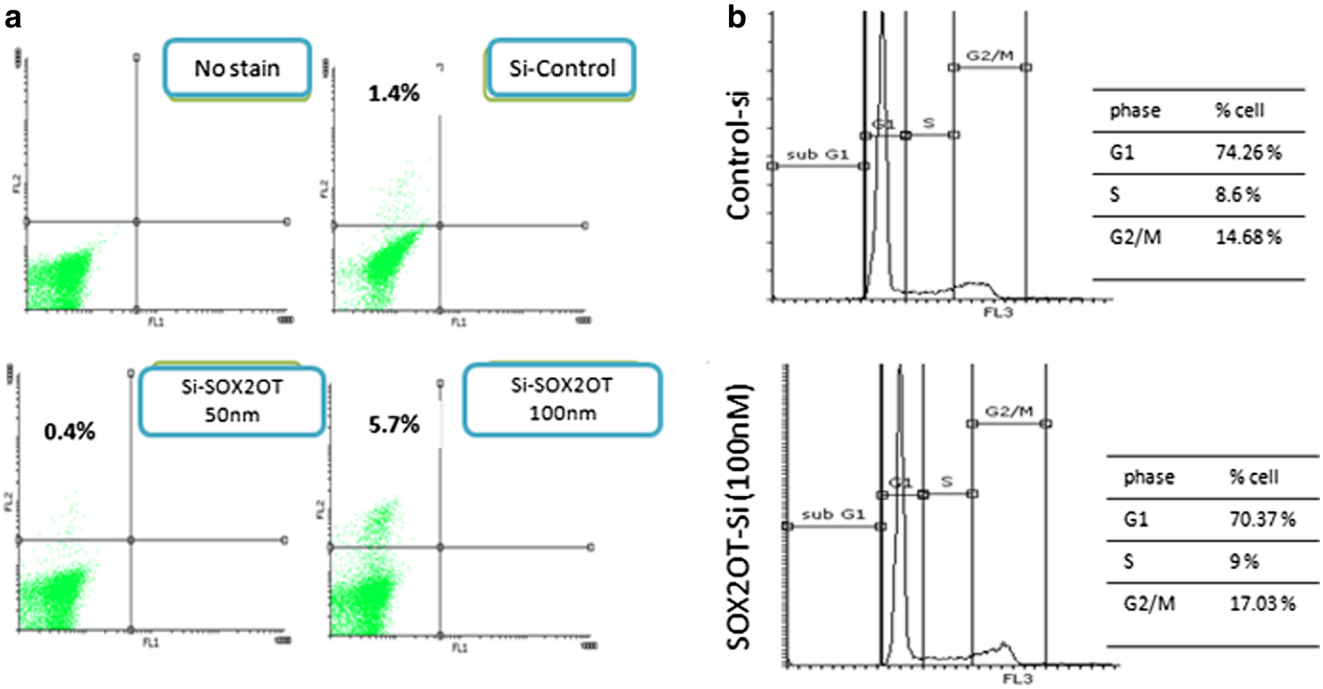

Fig. 5 The apoptosis (**a**) and cell cycle progression (**b**) of *SOX2OT* knocked down lung cancer cell line. The A549 cells were transfected with control and SOX2OT: siRNA (50 and 100 nM), and then were stained with annexin V/PI for apoptosis evaluation (**a**). As is shown, no annexin V-positive cells were detected. The percentage of necrotic cell (PI positive in *top left*) is shown in graphs. The flow cytometry evaluation of PI-stained cell cycle progression is illustrated in *right* (**b**). The percentage of cell population in each cell cycle phase is summarized for each treatment

spliced transcripts in lung tumors with exon junction specific primers. Our finding demonstrated over six folds upregulation of *SOX2OT* transcript variants 4 and 7 in >30 % of lung tumor samples compared to normal tissues. Similarly, we found that the magnitude of expression amplification of both *SOX2OT*s was more considerable in squamous cell carcinoma type of tumors that is concordant with Hou Z et al. report.

Because the *SOX2OT* encompasses *SOX2* gene [39] and the evidence of concordant *SOX2* expression with *SOX2OT* during stem cell differentiation and carcinogenesis, it has been

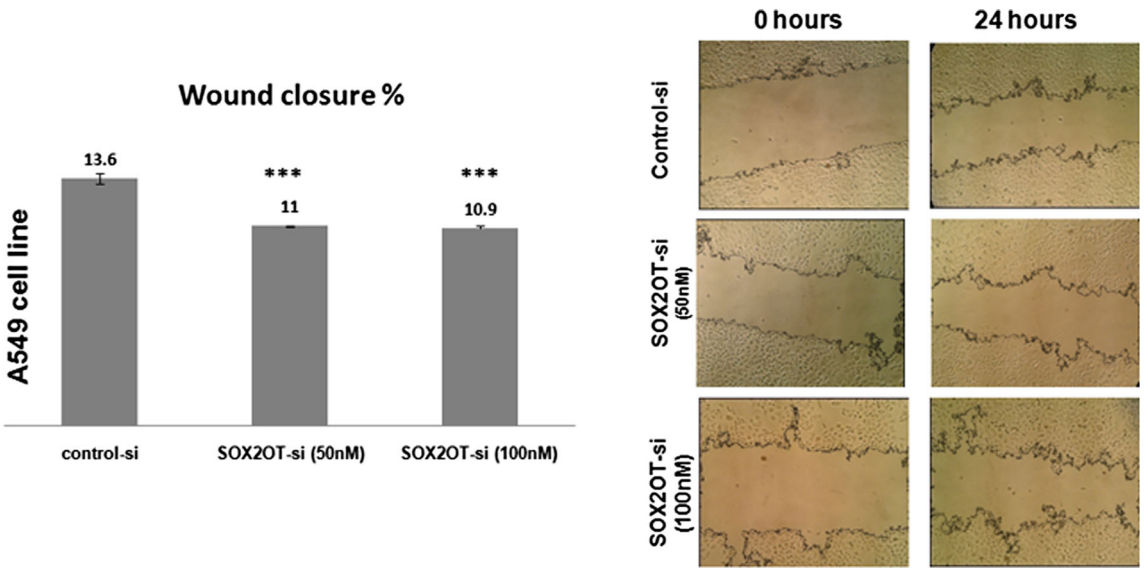

Fig. 6 *SOX2OT* knockdown effect on A549 wound closure ability. Cells were transfected with control siRNA or SOX2OT:siRNA (50 and 100 nM). The cells were imaged at 0 and 24 h after scratching. The wound edges were delimited by *black lines* and the wound area was measured for each field; then, the percent of wound closure was calculated

supposed that *SOX2OT* function is probably related to *SOX2* [18, 21, 23, 25]. In this study, we observed a significant correlation between *SOX2* and *SOX2OT* in lung tumor tissues and siRNA-treated cells which highlights the potential relevance of these two overlapping genes.

It has been reported that *SOX2OT* knockdown leads to G2/M arrest and proliferation inhibition in HCC827 and SKMES-1 lung cancer cell lines in a *EZH2* poly comb protein-dependent manner [38]. We found similar *SOX2OT* knockdown derived antiproliferative and cell cycle effect in A549 adenocarcinoma cancer cell line. An inhibitory function for SOX2OT suppression in hepatocellular carcinoma cell lines migration and invasion was reported previously [24]. In the current study, similar potential function for *SOX2OT* in A549 cell line migration was recorded. Although RNA interference [23, 25] and overexpression [21] analysis demonstrated that *SOX2OT* function is important in cancer cell, its exact function still remains to be investigated in detail.

In conclusion, the overexpression of *SOX2OT* transcript variants 4 and 7 in lung tumor tissues and its function in colony formation and proliferation of A549 cell line indicates the association of this long non-coding RNA with carcinogenesis and progression of non-small cell lung cancer. More investigation on *SOX2OT* function indeed will promote our knowledge on the molecular events happening during non-small cell lung tumor genesis and progression.

Acknowledgments This study was financially supported by Golestan University of Medical Sciences (grant number: 92041130) and Tracheal Diseases Research Center, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences.

Compliance with ethical standards

Conflicts of interest None

References

1. Cancer IAfRo. Latest world cancer statistics global cancer burden rises to 14.1 million new cases in 2012: marked increase in breast cancers must be addressed. World Health Organ. 2013(223):1–3
2. Dela Cruz CS, Tanoue LT, Matthay RA. Lung cancer: epidemiology, etiology, and prevention. Clin Chest Med. 2011;32(4):605–44.
3. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, et al. Cancer statistics, 2008. CA Cancer J Clin. 2008;58(2):71–96.
4. Cagle PT, Chirieac LR. Advances in treatment of lung cancer with targeted therapy. Arch Pathol Lab Med. 2012;136(5):504–9.
5. Heist RS, Sequist LV, Engelman JA. Genetic changes in squamous cell lung cancer: a review. J Thorac Oncol. 2012;7(5):924.
6. Birney E, Stamatoyannopoulos JA, Dutta A, Guigó R, Gingeras TR, Margulies EH, et al. Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project. Nature. 2007;447(7146):799–816.
7. Carninci P, Kasukawa T, Katayama S, Gough J, Frith M, Maeda N, et al. The transcriptional landscape of the mammalian genome. Science. 2005;309(5740):1559–63.
8. IHGS Consortium. Finishing the euchromatic sequence of the human genome. Nature. 2004;431(7011):931–45.
9. Enfield KS, Pikor LA, Martinez VD, Lam WL. Mechanistic roles of noncoding RNAs in lung cancer biology and their clinical implications. Genet Res Int 2012. 2012.
10. Xu G, Chen J, Pan Q, Huang K, Pan J, Zhang W, et al. Long noncoding RNA expression profiles of lung adenocarcinoma ascertained by microarray analysis. PLoS One. 2014;9(8):e104044.
11. Yanaihara N, Caplen N, Bowman E, Seike M, Kumamoto K, Yi M, et al. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell. 2006;9(3):189–98.
12. Yang J, Lin J, Liu T, Chen T, Pan S, Huang W, Li S. Analysis of lncRNA expression profiles in non-small cell lung cancers (NSCLC) and their clinical subtypes. Lung Cancer 2014.
13. Zhao W, Luo J, Jiao S. Comprehensive characterization of cancer subtype associated long non-coding RNAs and their clinical implications. Sci Rep. 2014;4:6591.
14. White NM, Cabanski CR, Silva-Fisher JM, Dang HX, Govindan R, Maher CA. Transcriptome sequencing reveals altered long intergenic non-coding RNAs in lung cancer. Genome Biol. 2014;15(8):429.
15. Qi P, Du X. The long non-coding RNAs, a new cancer diagnostic and therapeutic gold mine. Mod Pathol. 2013;26(2):155–65.
16. Rudin CM, Durinck S, Stawiski EW, Poirier JT, Modrusan Z, Shames DS, et al. Comprehensive genomic analysis identifies SOX2 as a frequently amplified gene in small-cell lung cancer. Nat Genet. 2012;44(10):1111–6.
17. Hussenet T, Dali S, Exinger J, Monga B, Jost B, Dembelé D, et al. SOX2 is an oncogene activated by recurrent 3q26.3 amplifications in human lung squamous cell carcinomas. PLoS One. 2010;5(1):e8960.
18. Amaral PP, Neyt C, Wilkins SJ, Askarian-Amiri ME, Sunkin SM, Perkins AC, et al. Complex architecture and regulated expression of the Sox2ot locus during vertebrate development. RNA. 2009;15(11):2013–27.
19. Mercer TR, Dinger ME, Sunkin SM, Mehler MF, Mattick JS. Specific expression of long noncoding RNAs in the mouse brain. Proc Natl Acad Sci. 2008;105(2):716–21.
20. Shahryari A, Jazi MS, Samaei NM, Mowla SJ. Long non-coding RNA SOX2OT: expression signature, splicing patterns, and emerging roles in pluripotency and tumorigenesis. Front Genet. 2015;6:196.
21. Askarian-Amiri ME, Seyfoddin V, Smart CE, Wang J, Kim JE, Hansji H, et al. Emerging role of long non-coding RNA SOX2OT in SOX2 regulation in breast cancer. PLoS One. 2014;9(7):e102140.
22. Iranpour M, Soudyab M, Geranpayeh L, Mirfakhraie R, Azargashb E, Movafagh A, Ghafouri-Fard S. Expression analysis of four long noncoding RNAs in breast cancer. Tumor Biol. 2015:1–8.
23. Shahryari A, Rafiee MR, Fouani Y, Oliae NA, Samaei NM, Shafiee M, et al. Two novel splice variants of SOX2OT, SOX2OT-S1, and SOX2OT-S2 are coupregulated with SOX2 and OCT4 in esophageal squamous cell carcinoma. Stem Cells. 2014;32(1):126–34.
24. Shi X-M, Teng F. Up-regulation of long non-coding RNA Sox2ot promotes hepatocellular carcinoma cell metastasis and correlates with poor prognosis. Int J Clin Exp Pathol. 2015;8(4):4008.
25. Hou Z, Zhao W, Zhou J, Shen L, Zhan P, Xu C, et al. A long noncoding RNA Sox2ot regulates lung cancer cell proliferation and is a prognostic indicator of poor survival. Int J Biochem Cell Biol. 2014;53:380–8.
26. Li X, Wang J, Xu Z, Ahmad A, Li E, Wang Y, et al. Expression of sox2 and oct4 and their clinical significance in human non-small-cell lung cancer. Int J Mol Sci. 2012;13(6):7663–75.

27. Kapranov P, Cheng J, Dike S, Nix DA, Duttagupta R, Willingham AT, et al. RNA maps reveal new RNA classes and a possible function for pervasive transcription. Science. 2007;316(5830):1484–8.
28. Gibb EA, Brown CJ, Lam WL. The functional role of long non-coding RNA in human carcinomas. Mol Cancer. 2011;10(1):38–55.
29. Prensner JR, Chinnaiyan AM. The emergence of lncRNAs in cancer biology. Cancer Discov. 2011;1(5):391–407.
30. Serviss JT, Johnsson P, Grandér D. An emerging role for long non-coding RNAs in cancer metastasis. Front Genet. 2014: 5.
31. Statt S, Thai P, Chen C-H, Cheng Y-W, Belinsky S, Wu R. Functional roles of SCAL1 expression in lung cancer and progression. Cancer Res. 2014;74(19 Supplement):1469.
32. Thai P, Statt S, Chen CH, Liang E, Campbell C, Wu R. Characterization of a novel long noncoding RNA, SCAL1, induced by cigarette smoke and elevated in lung cancer cell lines. Am J Respir Cell Mol Biol. 2013;49(2):204–11.
33. Gutschner T, Hämmerle M, Eißmann M, Hsu J, Kim Y, Hung G, et al. The noncoding RNA MALAT1 is a critical regulator of the metastasis phenotype of lung cancer cells. Cancer Res. 2013;73(3):1180–9.
34. Ji P, Diederichs S, Wang W, Böing S, Metzger R, Schneider PM, et al. MALAT-1, a novel noncoding RNA, and thymosin β4 predict metastasis and survival in early-stage non-small cell lung cancer. Oncogene. 2003;22(39):8031–41.
35. Schmidt LH, Spieker T, Koschmieder S, Humberg J, Jungen D, Bulk E, et al. The long noncoding MALAT-1 RNA indicates a poor prognosis in non-small cell lung cancer and induces migration and tumor growth. J Thorac Oncol. 2011;6(12):1984–92.
36. Tano K, Mizuno R, Okada T, Rakwal R, Shibato J, Masuo Y, et al. MALAT-1 enhances cell motility of lung adenocarcinoma cells by influencing the expression of motility-related genes. FEBS Lett. 2010;584(22):4575–80.
37. X-h L, Z-l L, Sun M, Liu J, Wang Z-x, De W. The long non-coding RNA HOTAIR indicates a poor prognosis and promotes metastasis in non-small cell lung cancer. BMC Cancer. 2013;13(1):464.
38. Hou Z, Zhao W, Zhou J, Shen L, Zhan P, Xu C, Chang C, Bi H, Zou J, Yao X. A long noncoding RNA Sox2ot regulates lung cancer cell proliferation and is a prognostic indicator of poor survival. Int J Biochem Cell Biol. 2014.
39. Fantes J, Ragge NK, Lynch S-A, McGill NI, Collin J, Howard-Peebles PN, et al. Mutations in SOX2 cause anophthalmia. Nat Genet. 2003;33(4):461–3.