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OCT4B1 Down-Regulates Self-Renewal Genes in Cancer Cell Lines

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ABSTRACT

Background: OCT4 is an important gene involved in stem cell (ESC, ASC and CSC) self-renewal and cell stemness states. The OCT4 gene encodes at least three variants, including OCT4A, B, and B1, each one has different biological activities. OCT4A acts as a silencing factor and downregulates cell differentiation. OCT4B is expressed in stem and somatic cells and does not play a role in cell stemness whereas OCT4B1 is expressed in stem cells, especially cancer stem cells, and has anti-apoptotic properties. The present study was carried out to investigate the relation between these variants and key genes in self-renewal pathways. ***Methods:*** Cancer cell lines from gastric adenocarcinoma (AGS), bladder tumor (5637), and brain tumor (132N1) were transfected by specific OCT4B1 siRNA using lipofection method and the gene was successfully suppressed in the test group after 48 hours. The expression of OCT4A, OCT4B, SOX2, NANOG and KLF4 genes were evaluated by qRT-PCR after this time. ***Results:*** The results demonstrated that expression levels of OCT4A and stemness genes including SOX2, NANOG and KLF4 were decreased following OCT4B1 downregulation, while expression of OCT4B was increased. ***Conclusion:*** Our results suggest a direct relation between expression of OCT4B1 and genes that control self-renewal. The data suggests that in the presence of OCT4B1, stemness genes are expressed, while in its absence, these genes will be down-regulated, therefore, strategies that specifically target repression could be considered for future molecular cancer therapies.

Introduction

OCT4 (octamer-binding transcription factor 4) belongs to a family of transcription regulatory genes containing the PUO DNA binding domain .[1] In human, the OCT4 gene is located on chromosome 6 and potentially encodes at last three different variants (A, B and B1) via mRNA alternative splicing.[1,2] OCT4 is one of the important and critical genes involved in embryonic and adult stem cell proliferation and differentiation that in collaboration with other genes such as SOX2, NANOG, and KLF4 (kruepple like factor 4) regulates self-renewal and the stemness state. [3,4] The expression of OCT4 induces stem cell pluripotency, while its silencing or downregulation is believed to be a sign of cell differentiation.[2,5]

There are different theories regarding the etiology of cancer and according to the most recent hypotheses "cancer stem cell", originates from either adult stem cells (tissue stem cells) or reprogrammed tissue somatic cells.[6]

Understanding the cellular and molecular pathways that trigger stem cell progression towards cancer stem cells would be useful for diagnosis, treatment, and prevention of cancers. Previous studied showed that OCT4 variants are important in cancer cell survival.[7] The expression of the OCT4A variant is exclusive to the stem cells (embryonic or adult), but not somatic cells, and regulates cell stemness. The function of OCT4B variant is unknown, although some studies showed that stress can cause upregulation of this variant.[8,9] Recently, Atlasi et al., reported that a new variant of OCT4, OCT4B1, was expressed in cancer tissues and cell lines.[1]

The OCT4B1 has anti-apoptotic properties [10] and its expression in cancer tissues and cell lines caused cell division and expansion of cancer tissue mass in the body. On the other hand, due to the anti-apoptopic properties of OCT4B1, a hypothesis was proposed suggesting that it can affect the expression profile of essential genes that control cell stemness, such as OCT4A, SOX2, NANOG, and KLF4.

This study aimed to suppress OCT4B1 by siRNA and subsequently evaluate the expression of OCT4A and B variants as well as SOX2, NANOG, and KLF-4 genes in AGS (adenocarcinoma), 5637 (bladder tumor), and 132N1 (brain tumor) cell lines.

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Material and methods

Cell lines and cell culture

The human cancer cell lines; AGS (gastric adenocarcinoma), 5637 (bladder tumor), and U-87MG (brain tumor) were obtained from the Iranian national cell bank (Pasteur institute of Iran, Tehran) and were subcultured in RPMI-1640 (Gibco) culture medium, supplemented by 100u/ml penicillin, 100µg/ml streptomycin, and 10% fetal bovine serum (FBS), at 37ºC in a humidified atmosphere of 5% CO_2.

Profiling of OCT4 variants

To measure mRNA expression of the OCT4 variants, total RNA was extracted from cultured cells (10^6 cells/ml) using TRIzol reagent (Invitrogen). To remove any DNA contamination, extracted RNA was treated by TURBO DNase I (Fermentase, Lithuania) and purity and integrity of RNA was measured by spectrophotometery (260/280 nm ratio) and gel electrophoresis (1% agarose gel) respectively.

First strand cDNA was synthesized at 42ºC for 60 min using 100 pmol oligo dT primer, 200 units of MMuLV reverse transcriptase and 1µg of total RNA according to the manufacturer's instructions (Fermentase, Lithuania).

Specific primers were designed for OCT4A, OCT4B, OCT4B1 (Figure 1) and ß-actin (Gene Bank accession numbers: NM-002701, NM-203289, EU518650 and NM-001101 respectively) using Gene Runner (version 3.02) and Allele ID (version 4.0) softwares (Table 1).

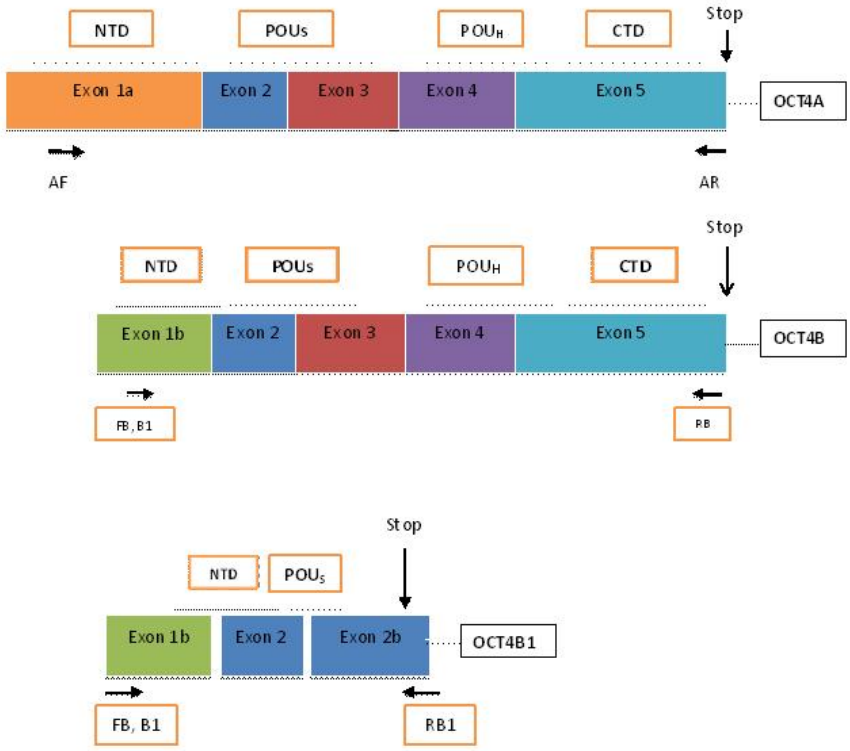


Figure 1. Schematic representation of exon organization and protein domains in OCT4 variants. OCT4B1 consists of all five exons, plus a novel exon, exon2b, flunked by exon2 and 3.Arrows show PCR primer positions. Primer set FB; B1/RB1 was used for sequence analysis of OCT4B1 variant. Abbreviations: CTD, C-terminal domain, NTD, N-terminal domain, POU_H, POU homeodomain, POU_S, POU-specific domain.

Quantitative Real Time PCR

Quantitative Real Time PCR was performed by addition of a SYBER green master mix (Parstous, Iran), 200ng of the generated cDNA, and 2 pg/µl of appropriate primers (Table 1). The following cycling program was set on a BIO-RAD CFX96 system (Bio-Rad Company, USA): one cycle at 95ºC for 15 min, 40 cycles at 95ºC for 30 S, 60ºC for 30 S (for OCT4B1 61ºC for 20 S) and 72ºC for 30 S. Real-Time PCR was carried out in triplicate and ß-actin was assessed as a house keeping gene for the normalization of amplification signals of the target genes. The proportional amounts of PCR products were determined using the $2^{-\Delta\Delta Ct}$ formula. The dissociation stages, melting curves, and quantitative analyses of the data were performed using CFX manager software version 1.1.308.111 (Bio-Rad, USA).

All PCR products were visualized by electrophoresis on a 1% gel containing 0.5 mg/ml ethidium bromide to check the size of PCR products.

Table 1. Designed primers for OCT4 variants, SOX2, NANOG, KLF4, and ß-actin.

Target genes	Designed Oligo	Relative Sequence	Fragment length
OCT4A	F	CGCAAGCCCTCATTTCAC	**111**
	R	CATCACCTCCACCACCTG	
OCT4B	F	AGAACCGAGTGAGAGGCAAC	**177**
	R	TGAGAAAGGAGACCCAGCA	
OCT4B1	F	GCACTTCTACAGACTATTCCTTGG	**128**
	R	TGATCCTCTTCTGCTTCAGG	
SOX2	F	TGCAGTACAACTCCATGACC	**164**
	R	GGACTTGACCACCGAACC	
NANOG	F	CCTATGCCTGTGATTTGTGG	**165**
	R	AGTGGGTTGTTTGCCTTTG	
KLF4	F	GGAGAGAGACCGAGGAGTT	**111**
	R	CTGACGCTGACGAGGACAC	
ß-actin	F	CACACCTTCTACAATGAGC	**160**
	R	ATAGCACAGCCTGGATAG	

OCT4B1 suppression

In order to suppress OCT4B1, two specific siRNA were utilized based on the sequence of exon2b (Figure 1). The primers are specific to sequences of OCT4B1 that distinguish this variant from A and B variants. Moreover, one irrelevant or scrambled siRNA (with no complementary target sequence in the human genome) was also used. The siRNA was designed by a selection program (whitehead institute for biomedical research, htt://jura.wi.mit.edu/) and the process of synthesis was performed by MWG Company (Germany) (Table 2).

For siRNA transfection, a suspension of freshly cultured cells was prepared (1×10^5 cells per millilitre) in the culture media without antibiotics and cultured in six well plates in tow group (test and control). During logarithmic growth phase (confluences 30-50%), cells were transfected with 50 nmol/ml synthetic siRNA using lipofectamin 2000 (Invitrogen, USA) and Opti-MEM media according to the manufacturers' instructions. In brief, 5µl of siRNA (25µM) and 4.5 µl RNAi-MAX reagents were diluted in 250 µl Opti-MEM and incubated for 10 min at room temperature. The mixture was then added to the cells in a final volume of 2.5ml. Cells were incubated at 37ºC in a humid atmosphere of 5% CO_2 for 72 hours.

MTT Assay

MTT assay depends on the ability of viable cells to metabolize MTT, a water-soluble tetrazolium salt, into a water-insoluble formazan product by mitochondrial succinate dehydrogenase.[11] Cell viability was analyzed 24, 48, and 72 hours after transfection. Briefly, $7x10^4$ cells per each well were seeded in a 96-well plate at a final volume of 200 µL. Following the cell transfection, the supernatant was replaced with 200 µL of warm RPMI 1640 (without phenol red). After adding 10 µL of 5 mg/ml MTT to each well, the plate was incubated in 37 ˚C for 3.5 h in the dark until a purple precipitate was visible under the light microscope. Then 100 µL of DMSO was added to each well and after 15 min the absorbance was read at 570 nm with a reference filter of 620 nm.

Apoptosis analysis

In order to measure the percentage of apoptotic cells in siRNA transfected cells (test group) compared to the scramble siRNA transfected cells (control group), 48 hours after transfection, single cell suspension were carried out and the Annexin V- FLOUS kit (Roch, Germany) was used to detect the exposed phosphatidylserin in the cell membrane, as described

elsewhere.[12] Briefly, 48 hours after transfection, cell lines (1×10^6) were centrifuged in eppendorf tubes and resuspended in 200µl of Binding Buffer. After 5 minute, Annexin V-FLOUS (1µl) and propidium iodide (1µl) were added to tubes and incubated at room temperature (5 minute) in the dark and then analyzed in a Beckman-Coulter Elite flow cytometer. Annexin V-FLOUS binding was detected using a FITC signal detector (FL1) and propidium iodide staining by the phycoerythrin emission signal detector (FL3). The percentage of FITC/PI positive cells (apoptosis) were calculated from flow histograms.[13]

Table 2. Sequences of designed siRNAs.

siRNA name	Target	Sequences
Version I	Target	**AAGGAGTATCCCTGAACCTAG**
	Sense	**(GGAGUAUCCCUGAACCUAG)dTdT**
	Anti-sense	**(CUAGGUUCAGGGAUACUCC)dTdT**
Version II	Target	**AAGAGGTGGTAAGCTTGGATC**
	Sense	**(CAGUGGUAAGCUUGGAUC)dTdT**
	Anti-sense	**(AAUCCAAGCUUACCACCUC)dTdT**
Scramble	Sense	**GCGGAGAGGCUUAGGUGUAdTdT**
	Anti-sense	**UACACCUAAGCCUCUCCGCdTdT**

Profile of OCT4A and B variants, SOX2, NANOG and KLF4 gene expression after suppression of OCT4B1

To detect the effect of OCT4B1 on the OCT4A and B variants, SOX2, NANOG and KLF4 gene expression, the cancer cell lines were cultured in test and control groups under similar conditions to 30-50% cell confluences and then OCT4B1 transcripts were suppressed in the test group by transfection of the siRNA as described above.

Following 48 hours of transfection, total RNA was extracted and cDNA was synthesized. Gene expression of OCT4A, B variants, SOX2, NANOG and KLF4 was measured by RT-Real-Time PCR using specific primers for each gene (Table 1).

Results

OCT4 variants were expressed in the cancer cell lines

The expression of all the OCT4 variants in the cell lines used in this study was evaluated. Specific primers for the A, B and B1 variants were designed and synthesized (primers were designed in a way to avoid nonspecific amplification of OCT4 pseudogenes, which have highly similar sequences to the A variant). ß-actin was used as housekeeping gene. Our data revealed that all three variants were expressed in the studied cell lines (Figure 2).

Transfection of cells with siRNA reduced the expression of OCT4B1

The expression of OCT4B1 was detected subsequent to 24, 48 and 72 hours of siRNA transfection in test and control group. In the test group, version I and II of designed siRNA and in the control group, scramble siRNA was used. The experiment was carried out in triplicate and the results showed that the version I siRNA transfection was more suppressive.. The data showed that optimal knockdown of the gene occurred after 48 hours of treatment (Figure 3).

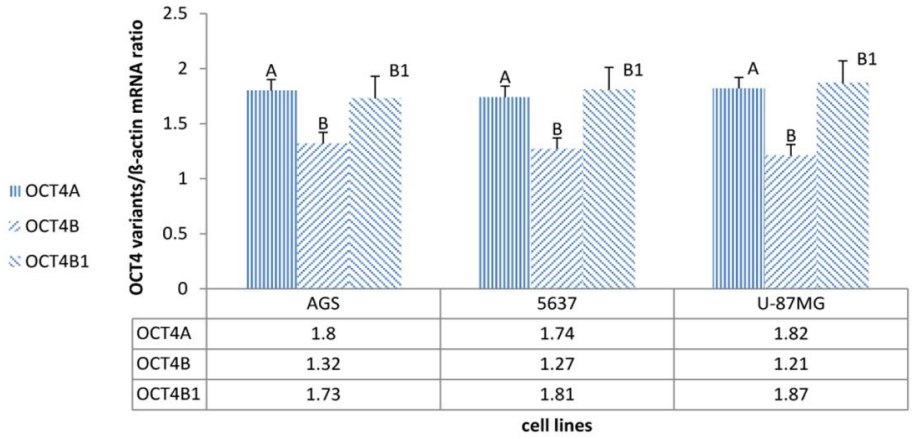
Apoptosis rate in cancer cell lines treated with specific OCT4B1 siRNA was increased

Flow cytometry analysis of the cells stained with Annexin V and PI demonstrated that up to 29% of the test group cells (transfected by specific OCT4B1 siRNA) had been undergone apoptosis three days after siRNA transfection compared to cells in control group (transfected by scramble siRNA).

MTT Assay showed reduction in cell activity after OCT4B1 siRNA transfection compared to the cells transfected by scramble siRNA (Figure 4).

OCT4B1 suppression reduced expression of OCT4A, SOX2, NANOG and KLF4

OCT4A, OCT4B, SOX2, NANOG and KLF4 gene expression were detected 48 hours after OCT4B1suppression in test group. The results indicated an increase in the expression of OCT4B but, the expression of the other studied genes was reduced when compared with the control group (Figure 5).



	AGS	5637	U-87MG
OCT4A	1.8	1.74	1.82
OCT4B	1.32	1.27	1.21
OCT4B1	1.73	1.81	1.87

Figure 2. Expression of OCT4 variants in cancer cell lines. The figure illustrates that OCT4A, OCT4B, and OCT4B1 are expressed in cancer cell lines.

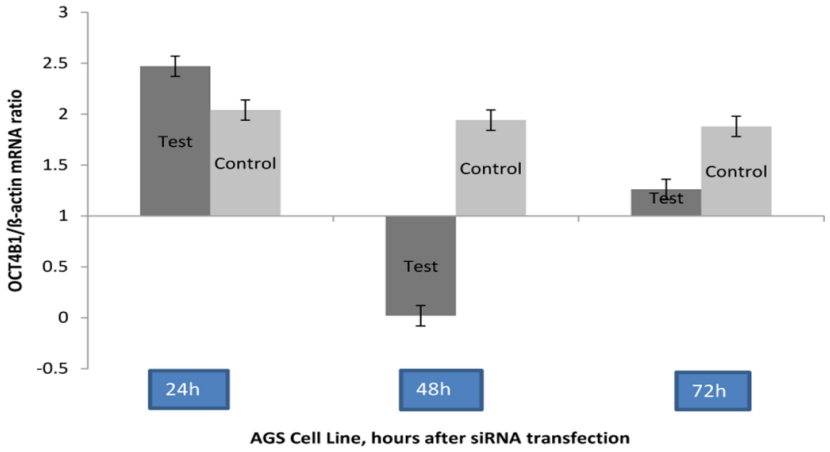
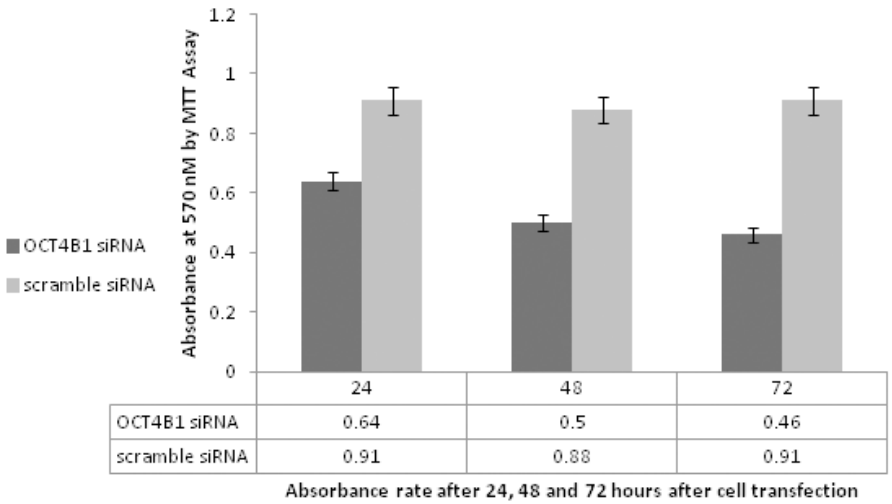


Figure 3. OCT4B1 expression after 24, 48 and 72 hours of siRNA transfection. The figure revealed that expression of OCT4B1 was reduced after 48h of siRNA transfection (p< 0.001).



	24	48	72
OCT4B1 siRNA	0.64	0.5	0.46
scramble siRNA	0.91	0.88	0.91

Figure 4. The surveival of 5637 tumor cell lines after transfection with OCT4B1 and scramble siRNA. Figure shows that OCT4B1 siRNA-transfected cells had lower viability compared to scrambled siRNA-transfected cells (p< 0.001).

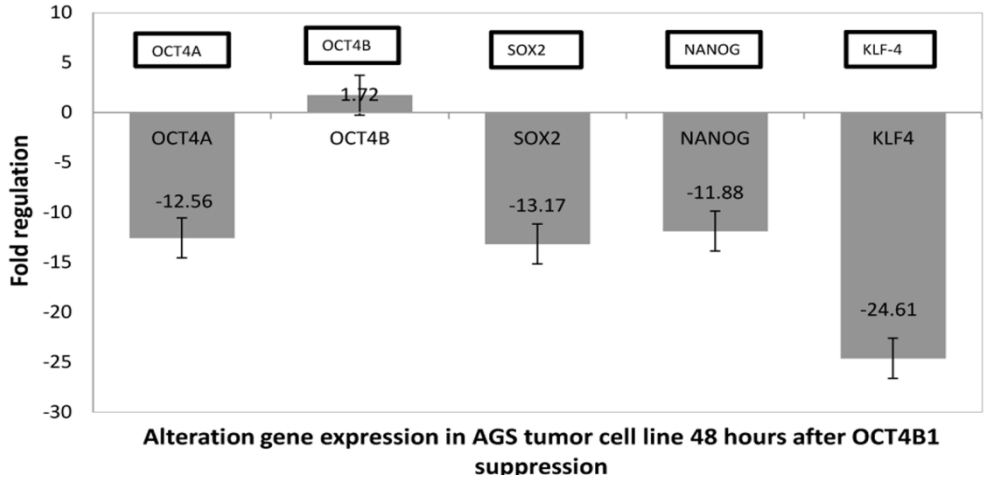


Figure 5. OCT4A, OCT4B, SOX2, NANOG, and KLF4 gene expression (fold changes) 48 hours after siRNA transfection (OCT4B1 suppression) in AGS tumor cell line. Figure shows increased expression of OCT4B and reduced expression of other genes.

Discussion

OCT4 is an important gene with transcriptional regulatory potency in stem cells[14] and in collaboration with SOX2, NANOG, KLF4 and some other genes, induces the stemness and self-renewal potential of stem cells.[15,18] OCT4 encodes at least three different alternatively spliced variants including A, B and B1.[1]

In contrast to somatic cells, OCT4 variants especially the OCT4B1 variant are expressed in cancer cell lines and cancer tissues.[9,10]

According to the latest hypothesis of cancer "cancer stem cell (CSC)", human adult stem cells (present in all tissues) or a reprogrammed somatic cell of tissues are origins of cancer. Two known properties of tumors, relapse and resistance to therapy, are among the most significant evidence for confirmation of current hypotheses on CSC.[6,19] It is now well established that in cancer cells and tissues, the OCT4B1 variant is expressed and studies have shown that this variant has anti-apoptotic effects on cancer cell lines.[10]

Due to a close relationship between OCT4, SOX2, NANOG, and KLF4 as master genes in the regulation of self-renewal state[4] and the stem cell state of cancer cells, based on cancer stem cell (CSC) hypothesis, the role of the OCT4B1 variant in the regulation of these genes was explored. Therefore, the OCT4B1 variant was suppressed and subsequently the expression levels of OCT4A, OCT4B, SOX2, NANOG and KLF4 genes were assessed.

Our results indicated that the expression of the OCT4A, SOX2, NANOG, and KLF4 were decreased after suppression of isoform (Figure 3). Therefore, according to our results it may be hypothesized that OCT4B1 can regulate expression of other important molecules including OCT4A, SOX2, NANOG, and KLF4 which are involved in the apoptosis pathways. Further investigations can determine how OCT4B1 downregulates these genes, in direct or indirect format.

Additionally, our results demonstrated that suppression of OCT4B1 have not led to alteration in expression of OCT4B variant. Interestingly, previous reports revealed that the OCT4B variant is not involved in stemness whereas it does contribute in cellular stress responses.[2] Therefore, it appears that expression of OCT4B is not under effects of OCT4B1 function.

OCT4A, which is the predominant variant of OCT4, is expressed in embryonic stem cells in the totipotent state and is downregulated subsequent to differentiation.[9] Some studies reported OCT4A expression in cancer cells.[20] Moreover, overexpression of SOX2 has also been observed in several cancer types.[5,21] This gene is an important factor with transcriptional activity that regulates the expression of several gene clusters involved in self-renewal potential.[22] The major role of NANOG is in embryonic stem cells and knocking out of this gene in early stages of mouse development leads to defective differentiation.[23] KLF4 is an essential gene with transcription factor activity in embryonic and adult stem cells and plays an important role in maintaining of stem cells self-renewal state. [25,26] Our results suggest that in presence of OCT4B1 variant OCT4A, SOX2, NANOG, and KLF4 genes will be expressed to maintain cell's self-renewal and immortalization. Thus, it seems that under normal growth conditions and the presence of OCT4B1, OCT4A and self-renewal master genes will be highly expressed, but when the OCT4B1 variant is suppressed, these genes which regulate immortalization would be downregulated.

Overall, the findings reported here indicated that after OCT4B1 suppression, the expression of OCT4A, SOX2, NANOG and KLF4 genes encoding proteins with transcriptional activity were decreased via unknown mechanisms and further studies can open new windows regarding the important roles played by OCT4B1 in the pathogenesis of tumors.

Conclusion

In conclusion, these results may shed light on how OCT4B1 facilitates its anti-apoptotic effects and show a relation between this variant and critical genes of the self-renewal pathway in stem cells. Therefore, knockdown or knockout of OCT4B1 could be considered for investigation of molecular mechanisms in cancer research.

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