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OCT4B1, a novel spliced variant of OCT4, generates a stable truncated protein with a potential role in stress response

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ABSTRACT

A novel variant of OCT4, OCT4B1, is highly expressed in pluripotent cells. OCT4B1 is also upregulated in various tumors and cell lines. Here, we have constructed a HA-tagged OCT4B1 construct, which could be translated into an N-terminally tagged protein, detectable by HA antibody. Western blotting revealed that OCT4B1 can act as a precursor for OCT4B, and it can also produce a truncated protein with a molecular size corresponding to that of predicted OCT4B1. Furthermore, our data demonstrated a cytoplasmic distribution for both isoforms. Interestingly, the expression ratio of OCT4B1/OCT4B transcripts and proteins was significantly elevated under the heat-stress condition.

1. Introduction

The octamer-binding transcription factor 4 (OCT4, also known as OCT3 and POU5F1; Entrez-Gene ID # 5460) is a master regulator of self-renewal and pluripotency in embryonic stem cells (ESCs) and embryonic carcinoma cells (ECCs) [1,2]. In these cells, the expression of OCT4 is quickly turned off upon the initiation of differentiation [3,4]. Accordingly, interfering with OCT4 expression in developing embryos or in cultured human or mouse ES and EC cells resulted in cell differentiation and loss of pluripotency [4–6]. In addition to the ESCs and ECCs, OCT4 expression has been also reported in germ cell tumors [7] and in various cancer cell lines and primary tumors [8,9]. However, its expression in somatic cells and tumors has been the subject of some controversies. The existence of several transcribed pseudogenes with high homology to the OCT4A sequence can be a potential source of false positive RT-PCR results. Another potential source of controversy is the presence of OCT4B and OCT4B1 spliced variants, the involvement of which in stem cell self-renewal still remains unclear [7,10,11].

The human OCT4 gene, located on chromosome 6 in the region of the major histocompatibility complex, can potentially encode three spliced variants, designated as OCT4A, OCT4B and the newly discovered OCT4B1 [12]. OCT4A and OCT4B isoforms share identical POU DNA-binding and C-terminal transactivation domains, but differ in their N-termini [13]. OCT4B1 is differed from OCT4B by retaining the intron 2, situated at the middle of POUs domain, as a cryptic exon (exon 2b). The insertion of the 233-bp cryptic exon causes a frame-shift within the sequence of the variant and the formation of a stop codon at the end of the cryptic exon [12]. Despite the great importance of the OCT4 expression in stem and cancer cells biology, few studies have so far

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tried to discriminate the differential expression and function of OCT4 spliced variants in these cells [14–16].

Based on the recent findings, OCT4A is the main variant responsible for the stemness properties in pluripotent cells [13]. The variant is primarily localized within the nucleus of the zygote, morola, inner cell mass of blastocysts, ESCs, ECCs, cancer stem cells, germ cells, and germ cell tumors; where it has a vital role in stemness property of the cells [14,17]. In contrast, OCT4B variant is mainly localized within the cytoplasm of somatic cells, cell lines and primary tumors, and cannot retain ESC's self-renewal [14,18]. Furthermore, a recent report indicated that OCT4B mRNA, containing a putative internal ribosome entry site (IRES), could be alternatively translated into at least three protein isoforms: OCT4B-265, OCT4B-190 and OCT4B-164. The OCT4B-190 has been reported to be involved in cell stress respond [19]. OCT4B1 is widely expressed in human pluripotent and nonpluripotent cells, and like OCT4A, is downregulated in differentiated cells. Therefore, OCT4B1 isoform could be considered as a putative marker for stemness [20]. The upregulation of OCT4B1 in gastric cancer, and also the elevated rate of apoptosis in AGS cells treated with OCT4B1-siRNA, indicated that the variant might have some roles in tumor initiation and/or progression [21].

Despite the emerging potential role of OCT4B1 in tumorigenesis, there is little information about the variant. Most importantly, it is not even known whether OCT4B1 can be translated as a stable truncated protein. For that reason, the main goal of the current research was to investigate the possibility of OCT4B1 protein(s) generation, and if it is so, to determine its sub-cellular localization.

2. Materials and methods

2.1. Cell culture and heat-shock conditions

The human embryonic kidney 293 (HEK-293) and USSC (unrestricted somatic stem cells) cells were obtained from the Stem Cell Technology Company (http://stemcellstech.com). Human bladder cancer cells (5637) were acquired from Iranian Pasture Institute. The human EC cell line NTERA2cl.D1 (NT2) was kindly provided by Dr. Peter Andrews, University of Sheffield. HEK-293 cells were cultured in DMEM with 10% fetal bovine serum (FBS) and 2 mM of glutamine (Invitrogen). 5637 and NT2 cells were cultured in RPMI-1640 (Gibco, USA) and high-glucose DMEM, respectively, both with 10% fetal bovine serum (FBS). USSC cells were cultured in low-glucose DMEM with 15% FBS and 2 mM of glutamine (Invitrogen). 5637 infected cells and NT2 were heat-shock treated at 45 °C for 1 h. The induction of OCT4B1 gene was conducted by using a final concentration 5 μl/ml (v/v) doxycycline (Sigma, USA) 24 h before heat- shock treatment.

2.2. Construction of FUW-tetO-OCT4B1-HA vector and transduction of 5637 and USSC cells

The corresponding sequence of human OCT4B1 was PCR-amplified partly from an OCT4A cDNA (exon 3–5) as well as a segment of human genomic DNA (exon2a and exon2b) using primers:

OCT4B1-Fragment1-Forward: 5′-AATCGGATCCATGCAC TTCTA CAG-3′.
OCT4B1-Fragment1-Reverse 5′-CATTGTTGTCAGCTTCCT CCAC-3′.
OCT4B1-Fragment2-Forward: 5′-GGAAGGTATTCAGCCA AACG-3′.
OCT4B1-Fragment2-Reverse: 5′-AGAATTCTCAGTTTGAA TGCATGGG-3′.

The PCR product was then cloned in pTZ57R/T plasmid (Fermentas, Lithuania) and the accuracy of the clone confirmed by DNA sequencing. Next, the OCT4B1-coding sequence was replaced the c-MYC sequence in a FUW-tetO-cMYC lentiviral vector, kindly provided by Dr. Hochedlinger [22]. Viral particles were then produced in HEK-293T cells, by co-transfection of lentiviral vectors, namely, FUW-tetO-OCT4B1 and rtTA, together with the packaging vectors, Δ8.9 and VSV-G. The viral containing supernatant was collected, passed through 0.45 μM filter, and concentrated by using Amicon ultrafilteration units (Millipore). About 200 μl of the concentrated viral particles were added to the target cells, in the presence of 5 μg/ml Polybrene (Sigma), the cells were incubated for 24 h in the presence of viruses. To induce OCT4B1-HA expression, 5 μg/ml Doxycycline was added to the media.

2.3. Reverse transcription (RT)-PCR and real-time PCR

Total RNA was extracted from the cells, using TRIzol reagent (Invitrogen, USA), according to the manufacturer's instructions. Purity and integrity of the extracted RNA was measured by UV spectrophotometry (260/280-nm ratio), as well as by visual observation of the samples stained with ethidium bromide and separated with agarose gel electrophoresis. Total RNA was digested with RNase-free DNase I (Fermentas, Lithuania) to remove any trace of DNA contamination. For each sample, a no-reverse transcription (No-RT) control was used in parallel from the DNase-treated RNA to detect any potential nonspecific amplification of genomic DNA. Reverse transcription was performed by first strand cDNA synthesis kit (Takara, Japan), followed by PCR amplification with a Taq DNA polymerase (Cinnagen, Iran). The sequences of the primers were as follows, as previously reported by Atlasi et al. [12]:

- Forward primer: 5′-AGACTATTCCTTGGGGCCACAC-3′
- Reverse primer: 5′-CTCAAAGCGGCAGATGG TCG-3′

The authenticity of the PCR products was then confirmed by DNA sequencing.

Specific primers and probes were designed for OCT4B, OCT4B1 and beta-2 microglobulin (b2m; GenBank accession numbers: NM_203289, EU518650, and NG_012920.1, respectively) using AlleleID 4.0 and Gene Runner software (Table 1). Real-time PCR was performed using Taqman method and premix Ex TaqTM kit (Takara) on an ABI 7500 real-time PCR system. PCR reaction mixture (12.5 μl premix Ex TaqTM, 0.2 μM forward primer, 0.2 μM

Table 1
The sequences and other characteristics of the primers and probes used to amplify and discriminate the expression of OCT4B1, OCT4B and internal control (b2m).

Gene	Designed oligo	Sequence	Amplicon length
OCT4B variant	F	AGATTGATAACTGGTGTGTTTATGTTC	177
	R	GCTGAATACCTTCCCAAATAGAAC	
	P	ATCCCAGGGTGATCCTCTTCTGCTTC	
OCT4B1 variant	F	GGGTTCTATTTGGTGGGTTCC	128
	R	TCCCTCTCCCTACTCCTCTTCA	
	P	ATTCTGACCGCATCTCCCCTCTAAG	
B2M	F	GGGTTTCATCCATCCGACATTG	123
	R	TGGTTCACACGGCAGGCATAC	
	P	TGCTCGCGCTACTCTCTCTTTCTGG	

F: forward primer, R: reverse primer, P: probe.

reverse primer, 0.1 μM TaqMan probe, 0.5 μl ROX reference dye II, 2 μl template, and 8 μl dH2O) was prepared and used for PCR under the following cycling conditions: initiation at 94 °C for 30 s, amplification for 45 cycles with denaturation at 94 °C for 5 s, annealing and extending at 60 °C for 34 s. To determine the reaction efficiencies for each primer pair and their corresponding probe, standard curves were plotted by using serial dilutions of the NT2′s cDNA. All reaction efficiencies were measured to be close to 100%. All experiments were conducted in duplicate or triplicate. Group-wise comparison and statistical analysis of relative expression of the target genes was carried out by REST 2008 (Relative Expression Software Tool, V2.0.7, Corbette Research Pty. Ltd.).

2.4. Immunocytochemistry

5637 and USSC cells were fixed in freshly made 4% paraformaldehyde for 15 min at room temperature. Cells were then permeabilized with 0.25% Triton X-100 [0.1% Tween-20 in phosphate buffered saline (PBS)] for 10 min and blocked in 1% BSA in PBST for 30 min. The cells were then incubated in the diluted primary antibody (mouse monoclonal anti-HA tag antibody, Sigma, USA, 1:5000) in PBST containing 1% BSA, for 1 h at room temperature. After washing the cells three times with PBS, cells were incubated with the secondary antibody (goat polycolonal anti-mouse antibody; Sigma, USA, 1:100 in 1% BSA) at dilutions 1:100 in 1% BSA for 1 h at room temperature. The cells were then washed three times with PBS and visualized under a fluorescent microscope (Nikon, Japan). For negative control samples, all conditions were kept the same, except for the omission of primary antibody.

2.5. Western blotting

An affinity-purified goat polyclonal anti-OCT4 antibody (sc-8629; Santa Cruz Biotechnology, USA, 1:1000) or a mouse monoclonal anti-HA tag antibody (Sigma, USA, 1:5000) were used for Western blotting. Luminescent signal was detected using the ECL Advance Western Blotting Detection Kit (Amersham, UK).

3. Results

3.1. Expression of an HA-tagged OCT4B1 construct in 5637 cell line generates two distinct transcripts and protein products

To investigate the possibility of any protein expression from OCT4B1 isoform, 5637 cell line was infected with viral particles containing OCT4B1 coding sequence. An immunogenic HA-tag had been fused at

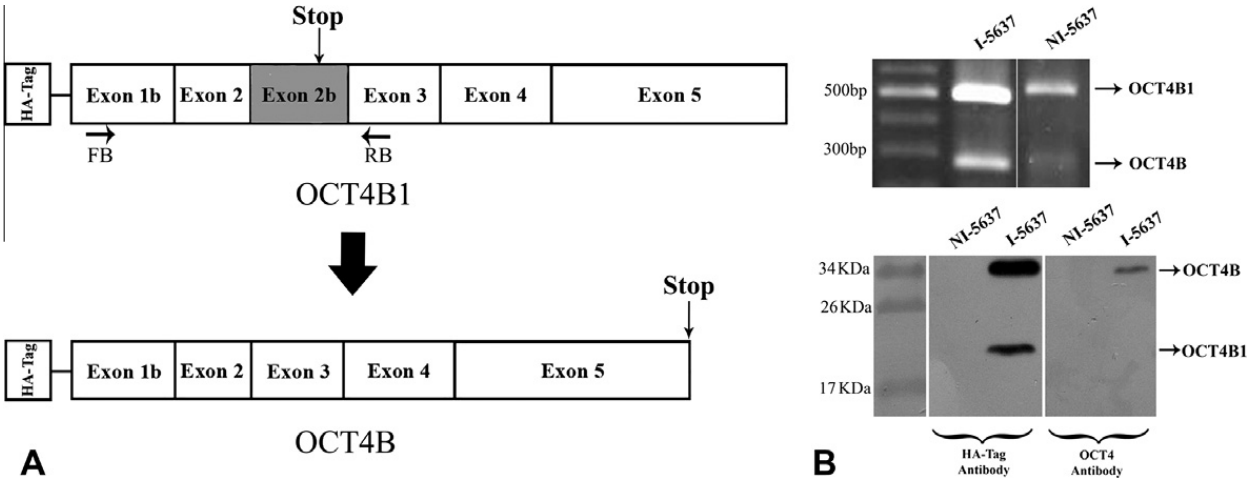

Fig. 1. The expression of HA-tagged OCT4B1 and OCT4B in bladder cancer cell line, 5637. (A) The schematic picture indicating the splicing of OCT4B1 to OCT4B. FB and RB primers were designed to discriminate the amplification of the two variants. The expected ORFs, terminating at the stop codons (vertical arrows), are exhibited. (B) Upper panel shows the RT-PCR amplification products in infected (I) and non-infected (NI) 5637 cells, with FB and RB primers. Lower panel shows the western blotting results of the same cells tested for the expression of OCT4 proteins by either an HA-tag or OCT4 antibodies. Note that OCT4 antibody cannot detect OCT4B1 isoform.

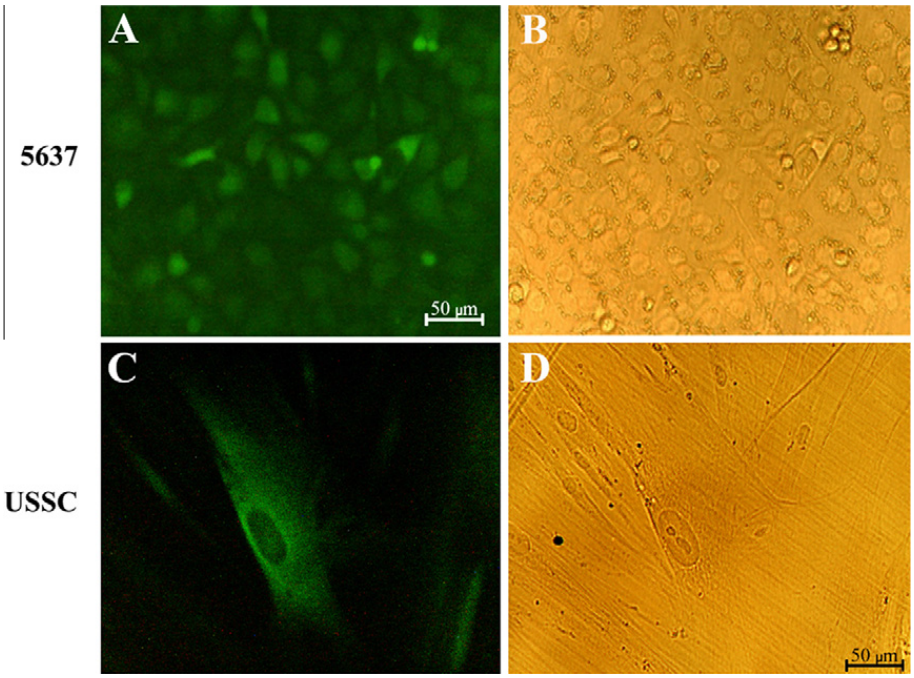

Fig. 2. Immunocytochemistry (ICC) was performed using an anti-HA antibody on 5637 (A) and USSC (C) cells. The results revealed a primarily cytoplasmic subcellular localization of the OCT4B1 protein products. (B and D) are the phase-contrast image of the same microscopic fields of (A and C), respectively.

the N-terminal part of the OCT4B1 (Fig. 1A). This tag allowed detection of any products of OCT4B1 that shares the N-terminal domain of the construct. Since OCT4B1 retains an extra intron segment (e.g. Exon2b; Fig. 1A), primers FB and RB can distinguish OCT4B1 from OCT4B (with a 494 bp and 267 bp PCR product sizes, respectively) [12]. As indicated in Fig. 1B (upper part), 5637 cells infected with FUW-tetO-OCT4B1-HA exhibited two PCR product bands, with the expected molecular sizes for both OCT4B1 and OCT4B variants. The finding was further confirmed by direct sequencing of the PCR products (data not shown). The expression level of these variants was then investigated, using quantitative qRT-PCR. Normalized to the endogenous levels of the transcripts in non-infected cells, the expression level of OCT4B1 and OCT4B variants increased by 1800 and 1400 times, respectively (data not shown).

To determine whether the generated transcripts of OCT4B1 and OCT4B can be translated into one or more protein products, western blotting was performed using two different antibodies. One antibody was against HA-tag, which could detect those products containing HA at their N-terminal domain; and the other one was against OCT4 that could detect OCT4A and OCT4B variants, but not OCT4B1. The predicted molecular weight of a putative OCT4B1 truncated protein and OCT4B variants plus their HA-tag, are 20 and 36 kDa, respectively. As it is shown in Fig. 1B, OCT4B1-HA construct primarily translated into a 36 kDa OCT4B-HA protein product, while to a lesser extent, it also generated a 20 kDa protein, corresponding to a truncated OCT4B1 protein.

As expected, the OCT4 antibody only detected OCT4B-HA, but not OCT4B1-HA isoform (Fig. 1B). The cell extract of non-infected 5637 cells were used as a negative control to distinguish between the background level and expression-induced level of protein production. Also, the cell extract of NT2 cells was used as a positive control to confirm the accuracy of antibody function.

3.2. OCT4B1-HA protein products are primarily located within the cytoplasm of infected cells

To determine the sub-cellular localization of the OCT4B-HA and OCT4B1-HA proteins, 24 h after the induction of infected 5637 cells, immunocytochemistry (ICC) was performed using a FITC-conjugated HA-tag antibody. Over 90% of the infected 5637 cells showed a cytoplasmic fluorescent signal, but the boundary between nucleus and cytoplasm of the cells was not clearly visible under the microscope (Fig. 2A). We also performed ICC on USSC cells which contain more expanded cytoplasm, compared to the 5637 cell line. ICC clearly showed a cytoplasmic immunoreactivity for OCT4B1-HA protein products in these cells (Fig. 2C).

3.3. The ratio of OCT4B1/OCT4B is altered in response to heat-stress condition

To determine whether the heat-stress conditions have any differential effects on the level of OCT4B1 and OCT4B production, the ratio of OCT4B1/OCT4B transcripts and proteins was determined in infected and non-infected 5637 cells, as well as in NT2 cells, as a control. The expression ratio of OCT4B1 to OCT4B transcripts was significantly changed under heat-stress condition. To determine the expression ratio of OCT4B1 to OCT4B proteins, western blotting was performed on 5637 infected and non-infected cells in both heat stress and normal conditions. The blotting was carried out by anti-HA-tag antibody and the results revealed a significant increase in the expression of OCT4B1-HA truncated protein, compared to OCT4B protein under stressed condition (see Fig. 3).

4. Discussion

OCT4 is a well-known stemness marker, believed to be exclusively expressed in embryonic stem cells. Recent reports on the expression of OCT4 in some somatic cells and various tumors ignited a hot debate on the authenticity of the data. Expressed OCT4 pseudogenes were the main suspect to blame for the controversial results [7,15,11]. Another reason for the conflicting reports on the subject could be due to the existence of OCT4′s alternatively spliced variants, i.e. OCT4A and OCT4B [23]. We have previously shown that OCT4A is primarily localized within the nucleus of the pluripotent cells, while OCT4B is localized within the cytoplasm of non-pluripotent cells [24]. The discovery of yet another variant in our lab, OCT4B1, made the matter more complex, as it is mostly expressed in pluripotent and undifferentiated cells, and turned off upon the induction of differentiation [12]. Indeed, a closer look at some previous reports indicated that the OCT4B1

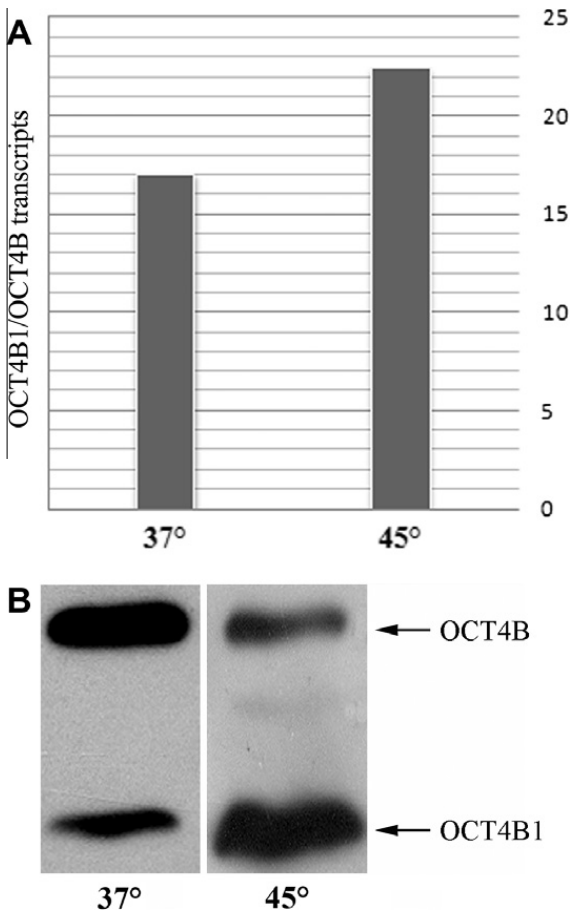

Fig. 3. Differential expression of OCT4B1 and OCT4B under normal (37°) and heat-stress (45°) conditions. (A) Real-time PCR results revealed a significant elevation in the relative expression of OCT4B1/OCT4B ratio under heat-stress condition. (B) The results of western blotting demonstrated a similar alteration in the OCT4B1/OCT4B ratio at protein level.

has been the variant identified in 59 hESC lines characterized by the International Stem Cell Initiative (ISCI), instead of OCT4A as previously assumed [20].

The recent reports on: (1) The correlation of OCT4B1 transcript's level with the state of cell differentiation [12]. (2) upregulation of OCT4B1transcript in gastric [21] colorectal [25] and bladder [26] cancers (3) an indirect role of OCT4B1, as the precursor of OCT4B, in stress-response and (4) an increased rate of apoptosis in a gastric cancer cell line treated with OCT4B1 siRNA [21] suggest a potential role for OCT4B1 in tumor initiation and progression. However, it is recently reported that the existence of a single nucleotide polymorphism (rs3130932) in the translation initiation codon of the OCT4B isoform, ATG → AGG [27], which is expected to hamper the expression of OCT4B and OCT4B1, had no significant association with breast, ovarian, and lung cancer risk [28]. Nevertheless, we still know little about the biological role of OCT4B1 in stem and tumor cells. Most importantly, it is not yet known if OCT4B1 could generate any stable protein isoform(s).

Due to the lack of a specific antibody against OCT4B1, we have generated an HA-OCT4B1 construct, in which the tag is fused to the N-terminal part of the OCT4B1 cDNA. We then followed the fate of expressed construct in a human bladder cancer cell (5637) and the unrestricted somatic stem cell (USSC) lines. Our data revealed that: (i) OCT4B1 could be served as a precursor to generate OCT4B transcript and protein. This data is in accordance with Gao et al., findings in which they report that introducing OCT4B1 cDNA into the mouse NIH3T3 cell line leads to the generation of three protein isoforms (i.e. OCT4B-265, OCT4B-190 and OCT4B-164) [29]. They explained that these isoforms are solely produced by further splicing of OCT4B1 into OCT4B. In contrary, our data demonstrated the existence of only one OCT4B isoform (corresponding to the main (OCT4B-265) isoform in Gao et al., report) generated from OCT4B1 translation. The latter discrepancy could be due to the usage of different cell lines and species in these separate investigations. (ii) In addition to the generation of OCT4B isoform, OCT4B1 transcript could also code for a truncated OCT4B1 isoform, generated by the insertion of a stop codon at the c-terminal side of the exon-2b. While the result clearly demonstrated the feasibility of a truncated HA-OCT4B1 protein generation in cell culture, the data needs further confirmation by raising a good specific antibody against OCT4B1 and examining the production of endogenous OCT4B1 isoform in different cell lines and tissues. (iii) The generation of OCT4B1 transcript and protein is elevated under heat-stress stimulation. This finding suggests a potential role of OCT4B1 in mediating stress response. This data is indirectly in agreement with the Wang et al. [19] findings, which introduced OCT4B as an anti-apoptotic factor under stress conditions. They also emphasized that the OCT4B isoform presented in the cells are generated from OCT4B1 splicing. Moreover, Asadi et al. [21] reported that OCT4B1 suppression under normal condition (with no stress) caused a significant rate of apoptosis in AGS cell line. Collectively, these reports attribute an anti-apoptotic characteristic to the OCT4B1 variant.

In conclusion, our previous and current findings suggest a functional and distinct role for OCT4B1 transcript and protein in stress-response and apoptosis. However, there is a need for further determination of endogenous level of OCT4B1 in different normal and tumoral tissues. The suitability of OCT4B1 as a potential tumor marker for detection and/or treatment of cancers is also another attractive experimental path to be followed in future.

Conflict of interest

None declared.

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