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Stem cell regulatory function mediated by expression of a novel mouse Oct4 pseudogene

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Abstract

Multiple pseudogenes have been proposed for embryonic stem (ES) cell-specific genes, and their abundance suggests that some of these potential pseudogenes may be functional. ES cell-specific expression of Oct4 regulates stem cell pluripotency and self-renewing state. Although Oct4 expression has been reported in adult tissues during gene reprogramming, the detected Oct4 signal might be contributed by Oct4 pseudogenes. Among the multiple Oct4 transcripts characterized here is a ~1 kb clone derived from P19 embryonal carcinoma stem cells, which shares a ~87% sequence homology with the parent Oct4 gene, and has the potential of encoding an 80-amino acid product (designated as Oct4P1). Adenoviral expression of Oct4P1 in mesenchymal stem cells promotes their proliferation and inhibits their osteochondral differentiation. These dual effects of Oct4P1 are reminiscent of the stem cell regulatory function of the parent Oct4, and suggest that Oct4P1 may be a functional pseudogene or a novel Oct4-related gene with a unique function in stem cells.
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Pseudogenes have traditionally been viewed as nonfunctional sequences of genomic DNA originally derived from functional genes due to their inability to be transcribed or translated into full-length proteins [1]. Processed pseudogenes, or retropseudogenes, arise by reverse transcription of processed mRNA followed by genomic integration [2]. A recent bioinformatic analysis reveals that retrotransposition frequency of embryonic stem (ES) cell-specific genes appears to far exceed than that of non-ES cell genes, and multiple highly homologous pseudogenes likely exist for all ES cell-specific genes [3,4]. For instance, 6, 16, and 10 pseudogenes have been proposed for human ES cell-specific genes Oct4, Stella, and Nanog, respectively, by nucleotide basic local alignment sequence tool (BLAST) searches against the respective gene mRNA transcripts [4]. Although the excessive frequency of these "theoretical" pseudogenes has been proposed to represent a potential signature event of ES cell-specific genes, whether any of these proposed pseudogenes may be functional remains to be determined.

Transcription factors containing the structurally bipartite POU domain are involved in the control of cell growth and differentiation in many tissues. Oct4 encoded by Pou5fl is a POU domain-containing transcription factor [5,6] that is involved in maintaining pluripotent and self-renewing state of ES cells [7,8]. Oct4 expression is normally restricted to pluripotent cells of the developing embryo, including ES cells and germ cells, and is down-regulated during ES cell differentiation [7,8]. Consistent with this demonstration, specific knockdown of Oct4 expression by RNA interference resulted in ES cell differentiation [9], and ectopic expression of Oct4 in somatic cells caused active cell dedifferentiation or tissue dysplasia [7,10]. Notably, genomic fusion between Pou5f1 and EWSR1 loci has been associated with a case of bone tumor, indicating translocation as a mechanism for Oct4 reactivation in cancer [11]. Along this line, expression of Oct4 can be activated during the process of carcinogenesis, indicating that abnormal expression of Oct4 could be oncogenic [12,13].

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Intriguingly, expression of Oct4 has been reported in adult stem cells and tissues based on the use of reverse transcription-polymerase chain reaction (RT-PCR) [12,14,15]. Since Oct4 pseudogene transcripts have not been characterized, the use of RT-PCR without DNA sequencing confirmation can potentially generate experimental artifacts regarding expression of Oct4. In addition, although often been viewed as junk genes, some pseudogenes that have been characterized are found to exhibit certain functional features [16,17]. To address these issues, we analyzed multiple full-length Oct4 transcripts, and found that Oct4 sequence-related transcripts are abundant in both embryonal carcinoma ES cells and non-stem cells. One of the Oct4 transcripts derived from P19 ES cells appears to be enriched in stem cells, and has the potential of encoding a truncated product. Adenoviral expression of this P19 Oct4 cDNA stimulates the proliferation of mesenchymal stem cells and inhibits their differentiation. These studies provide cautionary notes for analysis of expression of ES cell-associated genes, and indicate that some of the putative ES cell-specific pseudogenes may be functionally important.

Materials and methods

Cell culture. Porcine bone marrow-derived mesenchymal stem cells (MSCs) were isolated and cultured as described [18]. The MSC cultures used received less than 10 passages. C2C12 myoblasts [19], HEK293 cells [20], Sol8 muscle satellite cells [21], and P19 embryonal carcinoma stem cells [22] were maintained in MEM supplemented with 5% FBS. Cells were trypsinized using a 0.05% trypsin–0.5 mM EDTA solution upon confluency.

RT-PCR. RNA isolation and RT-PCR conditions were described previously [23]. The mouse Oct4 primers used for RT-PCR are derived from the GenBank mouse Oct4 cDNA sequence X52437 and NP_038661. We used two forward primers CGGGATGGATCCTCGAACCT and CCATGGCTGGACACCTGGCTTCA, and one reverse primer TGCCTCAGTTTGAATGCATG, which are expected to amplify ~1-kb full-length cDNA products. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers, which were described previously [23], yield a 0.98-kb product. Amplified products were cloned using the TOPO TA cloning system (Invitrogen, San Jose, CA, USA), and sequenced in both directions by the Roswell Park Cancer Institute DNA Sequencing Core.

Recombinant adenovirus. The recombinant adenovirus expressing Oct4P1 was constructed using the pAdEasy-1 system [24]. The cDNA was excised from the pCR cloning vector using *Kpn*I and *Xho*I, and inserted into the pShuttle-CMV vector. The recombinant shuttle DNA and pAdEasy-1 DNA were recombined in HEK293 cells. The virus was amplified three rounds in HEK293 cells to yield $\sim 10^9$ viral particles/ml culture medium. The control adenovirus Ad-LacZ as described previously [25] encodes a nuclear-localized β-galactosidase. For cell infection, viral lysates were diluted 10-fold with serum-free MEM, and added to adherent cells for 2–3 h with occasional agitation.

MTT assay. MSCs were plated onto 24-well plates (5000 cells/well) in growth media for the assays. Cells were infected after overnight adherence with Ad-LacZ and Ad-Oct4P1 virus in serum-free medium for 3 h, following which cells were kept in 0.3 ml growth medium supplemented with 2 μl virus per well (day 0). Cells were re-fed with fresh growth medium containing 2 μl virus per well on day 2. MTT assays were performed on day 0, 2, 3, and 4 as described previously [18]. MTT dye conversion was measured by absorbance at 540 nm.

Cell differentiation assays. For osteochondral differentiation, cells were plated on 35-mm dishes (1.5×10^5 cells/dish), and infected with adenovirus after overnight adherence. Infected cells were maintained in growth medium supplemented with 10 μl virus per dish for 2 days. Cells were then maintained in 1% FBS medium supplemented with 100 ng/ml bone morphogenic protein-7 (BMP7) for 10 days. Cells were washed twice with Hank's balanced salt solution and lysed in 0.1 ml phosphate-buffered saline containing 0.1% Triton-X 100. The cell lysates were used for enzyme and dye binding assays. Alkaline phosphatase activities were determined as described [18]. For dye binding assays, lysates were mixed with five volumes of 1% Alcian Blue solution prepared in 0.1 N hydrochloric acid at room temperature for 1 h. Precipitates were harvested by a 1-min centrifugation in a microfuge and extracted with dimethyl sulfoxide. Extracted dyes were measured by absorbance at 620 nm.

Western blotting. Total proteins were resolved by SDS–PAGE and electrotransferred to Immobilon-P membrane as described [23]. The membrane was incubated with a 1000-fold diluted primary antibody solution followed by washing with a saline solution supplemented with 0.025% TW-20. Secondary antibodies were conjugated with horse radish peroxidase. Signals were developed using the chemiluminescent substrate system (Pierce, Rockford, IL, USA) and imaged by Fuji imager. The monoclonal Oct4 antibody (clone C10) and PCNA antibody (clone PC10) were purchased from Santa Cruz (Santa Cruz, CA, USA). Protein concentrations were determined by the BCA protein assay method.

Results and discussion

Expression of Oct4, which is normally ES cell- and germ cell-specific, has been documented in adult stem cells and tissues based on analysis using RT-PCR [12,14,15]. However, these results cannot be adequately interpreted due to the presence of various Oct4 pseudogenes that may be transcriptionally active [26]. Although multiple Oct4 pseudogenes have been proposed based on bioinformatic analysis [4], their transcripts and functional significance have not been examined. To address these issues, we amplified and sequenced full-length Oct4 transcripts from multiple cell types using RT-PCR. Fig. 1 (top panel) shows that an Oct4 cDNA product of expected size (~1 kb) was amplified from mouse P19 ES cells, which is a pluripotent embryonal carcinoma stem cell line [22]. Interestingly, similar results were obtained from non-stem cell lines such as C2C12 myoblasts, HEK293 kidney cells, and Sol8 myoblasts. In sharp contrast to this result, Western blotting using a monoclonal Oct4 antibody detected a ~40-kDa Oct4 protein exclusively in P19 ES cells (Fig. 1, middle panel), confirming ES cell-specific expression of Oct4. Uniformly expressed proliferating cell nuclear antigens (PCNA) indicate that all cells examined here were in active cell proliferation phase (Fig. 1, bottom panel). Since Oct4 protein is a known ES cell marker, the non-ES cell transcripts detected by RT-PCR are most likely contributed by Oct4 pseudogenes that are transcriptionally active.

To characterize the detected Oct4 transcripts, we excised the ~1-kb RT-PCR products from the gel for DNA cloning and sequencing confirmation. Several clones were identified as listed in Table 1 along with their GenBank accession numbers. Among the multiple P19 ES cell-derived cDNA clones analyzed, the 957-bp cDNA (P19 clone 1) was most abundant, and was detected only in the ES cells. This Oct4 clone shares a ~87% sequence homology with the parent Oct4 gene encoding the 324-amino acid iso-

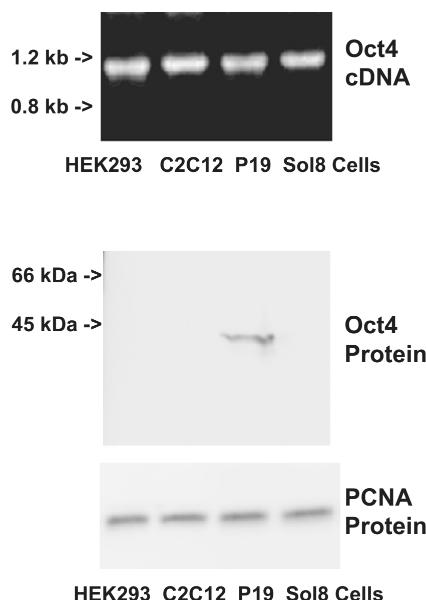

Fig. 1. Detection of multiple Oct4 transcripts and ES cell-specific Oct4 protein. Top panel: RT-PCR analysis of Oct4 transcripts isolated from P19 embryonal cells, HEK293 cells, C2C12 myoblasts, and Sol8 muscle satellite cells. The positions of two DNA marker bands (1.2 and 0.8 kb) were shown. DNA bands were revealed by ethidium bromide staining and imaged by Bio-Rad Gel Doc. Middle and bottom panels: Oct4 and PCNA proteins were detected by Western blotting. Protein bands were revealed by enhanced chemiluminescence and photographed by Fuji Imager. Twenty micrograms of proteins were applied per lane for SDS–PAGE.

Table 1
Amplified cDNA lengths and GenBank accession numbers

	cDNA length (no. of base pairs)	GenBank Accession No.
P19 clone 1	957	DQ851562
P19 clone 2	980	DQ851564
P19 clone 3	920	DQ851565
C2C12 clone 1	980	DQ851564
C2C12 clone 2	947	EF187444
Sol8 clone 1	959	DQ851563
Sol8 clone 2	980	DQ851564
HEK293 clone 1	1083	DQ851566

Note that P19 clone 1 (designated as Oct4P1) was only detected in P19 ES cells. P19 clone 2 is identical to C2C12 clone 1 and Sol8 clone 2.

form, and has the potential of encoding an 80-amino acid product (Fig. 2, top) possessing an N-terminal 26-amino acid homology with Oct4 [5]. Alterations in the coding sequence of this clone are caused by multiple point mutations, deletions, and insertions throughout the entire transcript. The predicted protein product has a basic isoelectric point (p$I = 10.02$). Although it exhibits no identifiable conserved protein domain when searched against the NCBI conserved domain database, using the PONDR (Predictor of Natural Disordered Regions) software, we found that it contains a central ordered core helical structure flanked by an N- and C-terminal disordered region. Additional clones were also identified in P19 ES cells. The 980-bp P19 clone 2 was detected in both P19 and non-ES cells (C2C12 clone 1 and Sol8 clone 2), and shares a ~88% sequence homology with the parent Oct4 gene. This clone represents the single most abundant Oct4 transcript in our RT-PCR analysis. The potential coding capacity of this clone is 34 amino acids.

The P19 clone 1, designated as Oct4P1 here, appears most interesting due to its abundant distribution in the mouse ES cells, although it may be classified as an Oct4 pseudogene. To determine whether expression of Oct4P1 might have some biological activity, we generated a recombinant adenovirus expressing Oct4P1 and examined whether it might affect the growth and differentiation phenotypes of mesenchymal stem cells (MSCs) [18]. RT-PCR analysis of infected MSCs showed the overexpressed Oct4P1 transcript in Ad-Oct4P1-infected MSCs only (Fig. 2, left panel). Proliferation of infected MSCs was then compared using MTT assays [18]. Fig. 2 (right panel) shows that overexpressed Oct4P1 stimulated the proliferation of MSCs 2 days after viral infection, which is reminiscent of the cell proliferation-promoting effect of Oct4 [7,10]. In line with this finding, MSCs expressing Oct4P1 exhibited a diminished ability to undergo osteochondral differentiation in response to BMP7, which is known to induce osteochondral differentiation of MSCs and other progenitor cells [27,28]. Fig. 3 (left panels) showed that formation of BMP7-induced MSC osteochodrocytic nodules was suppressed by Oct4P1 expression. Biochemical assays show the activity of the osteogenic enzyme alkaline phosphatase was decreased by ~50% in the presence of Oct4P1 (Fig. 3, middle panel). Chondrogenic synthesis of sulfated proteoglycans as monitored by Alcian Blue binding was decreased by ~75% in Oct4P1-expressing MSCs (Fig. 3, right panel).

Analysis of the GenBank database reveals that at least three mouse Oct4 transcripts have been cloned encoding proteins of 324 [5], 352 [29], and 377 amino acids [30], and three human Oct4 transcripts encoding proteins of 164 (Accession No. BC020712), 265 and 360 amino acids [31]. Oct4P1 has the potential of encoding an 80-amino acid protein, and thus represents the shortest protein characterized within the Oct4 family. Similar to other members of the family, Oct4P1 transcript is preferentially enriched in P19 ES cells, and may also play a dual role in regulating self-renewal and pluripotent differentiation of ES cells. For instance, expression of BMPs has been found to be inhibited by Oct4 [32,33], and we show that overexpression of Oct4P1 antagonizes the osteochondral differentiation pathway mediated by BMP7. Although P19 ES cells are derived from teratocarcinomas, these cells are closely related to embryonic stem cells [22,34]. Nonetheless, it will be interesting to determine whether Oct4P1 transcripts may also be abundantly present in primary mouse and human ES cells.

Expression of Oct4 has been reported in adult stem cells and tissues based on the use of RT-PCR [12,14,15]. Pluripotency mediated by gene reprogramming has also been

1
MDPRTWLSFQGPPGGPGIGPGSEVLGSPHVHRHMSSAEGW
HTVDLRLAWRLCSLRARQEQEWRATQKKPSLGLVPTAPVP
80

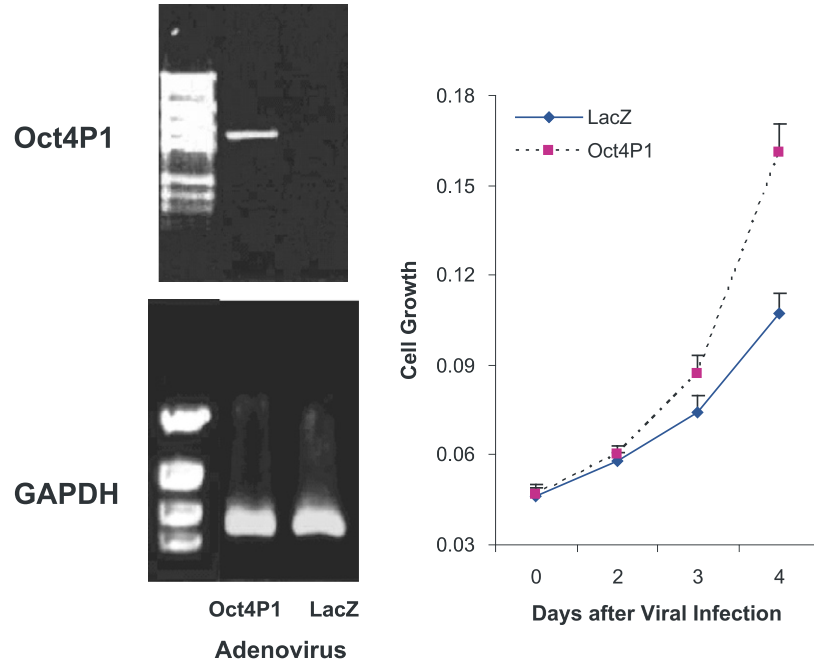

Fig. 2. Adenoviral expression of Oct4P1 and its effect on MSC proliferation. The predicted amino acid sequence of Oct4P1 was shown on the top. Left panels: MSCs were infected with Ad-LacZ and Ad-Oct4P1. Total RNA was isolated 2 days after infection, and analyzed by RT-PCR using Oct4 and GAPDH primers. The top four DNA size marker bands (left lane) are 4.5, 1.9, 1.2, and 0.8 kb. Right panel: MTT cell proliferation assays of adenovirus-infected MSCs. Cell growth was monitored by absorbance at 540 nm expressed as means ± standard errors. $P < 0.01$ and 0.005 comparing LacZ and Oct4P1 at day 3 and day 4, respectively.

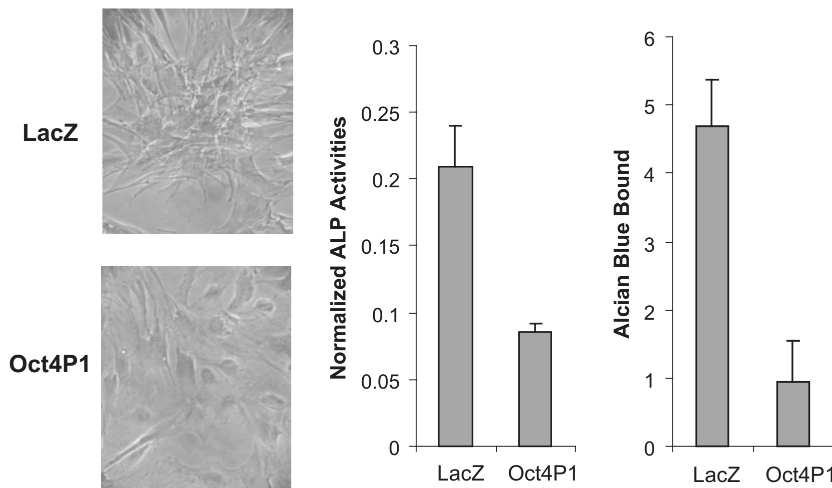

Fig. 3. Inhibition of MSC osteochondral differentiation by Oct4P1. Right panels: Phase contrast images of adenovirus-infected MSCs exposed to BMP7 for 10 days. BMP7 treatment induced cell aggregation and osteochondrocytic nodule formation in Ad-LacZ-infected MSCs only. Middle panel: Activities of the osteogenic enzyme alkaline phosphatase monitored by absorbance at 412 nm per μg proteins, $P < 0.01$. Right panel: Chondrogenic synthesis of acidic proteoglycans expressed by Alcian Blue dye retention (absorbance at 620 nm) per μg proteins, $P < 0.01$.

shown to be associated with Oct4 reactivation [35]. However, these studies could not exclude the possibility that the detected Oct4 signal came from Oct4 pseudogene transcripts. As shown here, the use of RT-PCR for analysis of ES cell-specific gene expression needs to be interpreted with caution, and be accompanied by DNA sequencing

to confirm the identity of the detected Oct4 transcript(s). Proper design of Oct4 clone-specific PCR primers and development of clone-specific antibodies should prove valuable in studying the expression and function of Oct4 gene members.

Expression of ES cell-specific genes Oct4 and Nanog are known to be required for or promote ES cell proliferation [7,10,36]. Our finding based on adenoviral expression of Oct4P1 in MSCs indicates that this property of Oct4 and Nanog may similarly operate in adult mesenchymal stem cells, suggesting that ES and adult stem cells may share overlapping mechanisms governing their growth and differentiation. Consistent with this view, ectopic activation of Oct4 causes the expansion of adult progenitor cells in mice [10]. Thus, Oct4P1 in spite of its coding sequence truncation preserves at least some aspect of the parent Oct4 function. The presence of a large number of potential pseudogenes for ES cell-specific genes [4] suggests that some of these pseudogenes may function in an unknown fashion. Indeed, although pseudogenes are traditionally viewed as non-functional due to their inability to translate full-length proteins, some pseudogenes are known to possess certain regulatory roles when examined [16,17]. It has been proposed that pseudogenes be considered as potogenes, i.e., DNA sequences with a potentiality for becoming new genes [2]. In this context, Oct4P1 may be viewed as a functional Oct4 pseudogene or a novel Oct4-related gene with a unique function in stem cells.

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