Original Paper

Cells Tissues Organs

Cells Tissues Organs 2006;184:105–116
DOI: 10.1159/000099617

Accepted after revision: October 5, 2006

Isolation and Characterization of a Population of Immature Dental Pulp Stem Cells Expressing OCT-4 and Other Embryonic Stem Cell Markers

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Key Words
Dental pulp stem cells · Embryonic stem cell markers · Engraftment · Spontaneous differentiation · Stem cell therapy

Abstract
We report the isolation of a population of immature dental pulp stem cells (IDPSC), which express embryonic stem cell markers Oct-4, Nanog, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 as well as several other mesenchymal stem cell markers during at least 25 passages while maintaining the normal karyotype and the rate of expansion characteristic of stem cells. The expression of these markers was maintained in subclones obtained from these cells. Moreover, in vitro these cells can be induced to undergo uniform differentiation into smooth and skeletal muscles, neurons, cartilage, and bone under chemically defined culture conditions. After in vivo transplantation of these cells into immunocompromised mice, they showed dense engraftment in various tissues. The relative ease of recovery and the expression profiles of various markers justify further exploration of IDPSC for clinical therapy.

Abbreviations used in this paper

ACSP	anti-chondroitin sulfate proteoglycan
AG	aggrecan
AP	alkaline phosphatase
CH	Chinese hamster
DMEM	Dulbecco's modified Eagle's medium
DPSC	dental pulp stem cells
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GBA	Goshe syndrome gene
GFAP	glial fibrillary acidic protein
IDPSC	immature dental pulp stem cells
KSR	Knockout Serum Replacement
mMAPC	mouse bone marrow multipotent adult progenitor cells
Oct-4	POU transcription factor Octamer-4
PBS	phosphate-buffered saline
PI	propidium iodide
RA	retinoic acid
SHED	stem cells from human exfoliated deciduous teeth
SLS	sphere-like structures
SSEA	stage-specific embryonic antigen
TRA	tumor recognition antigen

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Introduction

As an alternative to embryonic stem cell therapy, adult stem cells have shown promise [Caplan, 1991, 2000, 2003, 2004, 2005; Caplan and Bruder, 2001; Kuehle and Goodell, 2002; Pittenger, 2004]. For example, multipotent adult progenitor cells from mouse bone marrow (mMAPC) were shown to express several embryonic stem (ES) cell markers, such as Oct-4 (POU transcription factor), Rex-1 (transcription factor) and SSEA-1 (stage-specific embryonic antigen), and to contribute to all embryonic cell lineages when a single cell is injected into the blastocyst [Jiang et al., 2002]. While bone marrow is an excellent source of stem cells with proven therapeutic value, the process of collecting bone marrow is invasive, and, moreover, recent data implicate bone marrow stem cells in cancer development [Houghton et al., 2004]. The expansion of the list of the potential sources of pluripotent adult stem cells beyond a small group consisting of cord blood, bone marrow, adipose tissue, and amniotic stem cells [Jiang et al., 2002; Zuk et al., 2002; Miki et al., 2005] would be of value.

Extending earlier findings in rodents [Mann et al., 1996], the recent discovery of relatively immature stem cells in the dental pulp of human exfoliated deciduous teeth (SHED) has offered a potentially non-invasive source of stem cells [Miura et al., 2003]. SHED showed rapid expansion and proliferation in vitro while expressing several mesenchymal stem cell markers, such as STRO-1 and CD146. Stem cells from dental pulp [Miura et al., 2003] appeared to be inferior in their potential therapeutic value compared to ES cells or mMAPCs, since they were not shown to express Oct-4, SSEAs, Nanog, or any other hallmarks of totipotent stem cells, while their multilineage terminal differentiation was only marginally successful [Jiang et al., 2002; Chambers et al., 2003; Constantinescu, 2003; Laslett et al., 2003; Mitsui et al., 2003; Pierdomenico et al., 2005; Laino et al., 2006]. SHED have been shown to be highly heterogeneous, because only 9% of SHED express markers of undifferentiated cells, and it is not clear if clones obtained from SHED maintain expression of these markers [Miura et al., 2003].

Previously, it has been reported that removal of stem cells from their natural milieu may change their differentiation properties [Bissell and Lafarge, 2005; Schwartz and Verfaillie, 2005]. Therefore, we attempted to recover stem cells using outgrowth culture as the initial step in order to preserve as much as possible the natural cellular environment. The dental pulp stem cells (DPSC) described here were characterized by their ability to proliferate indefinitely without differentiation, by marker proteins and RNA expression, by multiple lineage differentiation under chemically defined culture conditions, and by their ability to engraft in vivo.

Materials and Methods

Cell Culture

Dental pulp was extracted from normal exfoliated human deciduous teeth of 5- to 7-year-old children (10 patients) under local anesthetic at the Dental Clinic CERA with informed consent of the patients. Dental pulp was pulled out with a barbed Nervbroach (dental instrument), washed twice with sterile phosphate-buffered saline (PBS; 0.01 M, pH = 7.4) supplemented with antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), and transferred (with minimal dissection) into 35-mm Petri dishes (Corning, New York, N.Y., USA) with Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 (1:1, Invitrogen, Carlsbad, Calif., USA) supplemented with 15% fetal bovine serum (FBS, HyClone, Logan, Utah, USA), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 2 mM nonessential amino acids. Tissue explant of dental pulp was used to isolate immature DPSC (IDPSC). The growing culture of IDPSC was maintained under these conditions for 2 weeks, and then the cells were washed twice in PBS, dissociated in a 0.25% trypsin solution (Invitrogen), and seeded at 10^4 cells per 25-cm^2 flask. The culture was maintained semiconfluent in order to prevent the differentiation of the cells, and the cells were passed every 4–5 days with the medium refreshed daily. For freezing, cells were resuspended in a medium containing 20% FBS, 70% DMEM, and 10% dimethylsulfoxide (Sigma, St. Louis, Mo., USA) at 5×10^5 cells/ml, and the temperature was slowly and gradually decreased at a rate of 1°C per minute until a final temperature of –70°C was reached. Thereafter, cells were transferred to liquid nitrogen. For thawing cryo-vials with IDPSC, they were placed into a 37°C water bath for 2 min and thereafter washed twice with 20% FBS and 70% DMEM, and placed into culture.

Early passage human adult skin fibroblasts with normal karyotype were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (Invitrogen). All cultures were incubated at 37°C in a 5% CO_2 and high humidity environment.

Analysis of Cloning Efficiency and Growth Curves

To conduct cloning efficiency experiments, IDPSC were dissociated into single cells, the cell number determined, and 10^2 cells were plated in a Petri dish (90 × 15 mm). The resulting colonies were counted 6 days after plating. The experiments were repeated three times with wells from teeth from 3 different donors.

To analyze growth kinetics of IDPSC, single clones were isolated with cloning rings and expanded. When each clone reached 10^5, cells from four separate clones corresponding to four IDPSC cell lines were harvested every 2nd day (for 16 days), trypsinized, and counted. Three separate growth experiments were performed for each clone.

In vitro Differentiation

For all types of differentiation, DMEM^{Knockout} medium supplemented with 20% Knockout Serum Replacement (KSR; Invitrogen), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine was used. Neural differentiation was induced as previously described [Fraichard, et al., 1995] by all-trans retinoic acid (RA, Sigma) or dimethylsulfoxide, and neuronal cells were maintained in Neurobasal (Invitrogen) culture medium supplemented with B27 (Invitrogen). For sphere-like structure (SLS) formation, 35-mm Petri dishes pre-treated with 0.1% agarose solution (Sigma) were used. The details of cell differentiation are presented in the respective parts of the manuscript.

Antibodies and Immunocytochemistry Analysis

The ES Cell Marker Sample Kit for the detection of stage-specific embryonic antigens (SSEA-3 and SSEA-4), tumor recognition antigens (TRA-1-60 and TRA-1-81), and Oct-4 was purchased from Chemicon (Temecula, Calif., USA). Antibodies against SH-2, SH-3, and SH-4 were from Case Western Reserve University, Cleveland, Ohio, USA. Mouse monoclonal nestin and glial fibrillary acidic protein (GFAP) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif., USA), β-tubulin III and α-actinin (sarcomeric) antibodies from Sigma, and antibodies to titin and smooth muscle actin, anti-chondroitin sulfate proteoglycan [ACSP, aggrecan (AG)] and anti-fetal cartilage proteoglycan from Chemicon. The following immunostaining protocol was used: IDPSC growing on coverslips were washed twice in rinse buffer (20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.05% Tween-20), fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. After blocking with 5% bovine serum albumin, the cells were incubated with diluted primary antibodies for 1 h at room temperature. Primary antibodies from the ES Cell Marker Sample Kit were diluted 1:40 and 1:100. After washing three times in rinse buffer, fluorescein isothiocyanate (FITC)- or Cy3-labeled appropriate secondary antibodies were added for 30 min at a 1:100 dilution. Undifferentiated IDPSC were stained for alkaline phosphatase (AP) as described before [Talbot et al., 1993]. To analyze osteogenic differentiation, fixed cells were incubated for 30 min in the dark with a mixture containing naphthol AS-MX AP solution and fast red violet salt [Lan et al., 2003]. For von Kossa staining, a standard protocol was used [Sheehan and Hrapchak, 1980]. Microscope slides were mounted in Vectashield mounting medium (Invitrogen) with or without DAPI. Analysis was performed with digital images that were acquired with a cooled CCD camera (PCO, VC44) and processed with ISIS software (MetaSystem, Belmont, Mass., USA).

Karyotype Analysis

IDPSC were grown to subconfluence, and demecolcine (Sigma) was added to a final concentration of 0.1 μg/ml for 1 h. Cells were then trypsinized, pelleted by centrifugation at 1,000 rpm for 5 min, resuspended in 5 ml of 0.075 M KCl, and incubated for 15 min at 37°C. Cells were pelleted by centrifugation, the supernatant was removed, and ice-cold fixative solution composed of 1 part acetic acid and three parts methanol was added to a final volume of 10 ml. The fixative solution was changed twice followed by centrifugation. Metaphases from cells were analyzed by G-banding. At least 250 metaphase spreads were counted and 50 metaphases analyzed for each IDPSC line. Karyotypes were analyzed according to the 'International System for Human Cytogenetic Nomenclature' on a Zeiss II microscope (Zeiss, Jena, Germany).

Flow-Cytometric Analysis

For flow-cytometric analysis, the following antibodies against cell surface molecules and their respective isotype controls were used: monoclonal anti-human CD45, CD13 (Sigma), CD43, and CD34 (BD-PharMingen, San Diego, Calif., USA) and CD105 (Serotec, Oxford, UK). About 10^6 cells were incubated with primary antibody for 30 min on ice, washed in PBS + 2% FBS and 1 μM sodium azide (buffer) followed by addition of secondary FITC- or phycoerythrin-conjugated antibody. Proliferative activity of IDPSC was analyzed with FITC-conjugated proliferating cell nuclear antigen (Chemicon). For staining of intracellular antigens, the cells were fixed and the following protocol of permeabilization was used. The pellet was resuspended in 1 ml of Tween-20 solution (0.2% in PBS) at room temperature, and the mixture was incubated for 15 min in a 37°C water bath. Flow-cytometric analysis was performed on a fluorescence-activated cell sorter (FACS, Becton, Dickinson, San Jose, Calif., USA) with the CELL Quest program (Becton, Dickinson).

RT-PCR

Total RNA was extracted with Trizol reagent (Invitrogen) and reverse transcribed with the ProSTAR First-Strand RT-PCR kit (Stratagene, La Jolla, Calif., USA) and oligo$(dT)_{12}$ according to the manufacturer's instructions. In each experiment, 5 μg of total RNA were used for RT in a final reaction volume of 50 μl, with 1 μl being used for PCR. The following primers were used for amplification by RT-PCR: *Rex1,* transcription factor, forward primer 5′GGTGAGTTTTCYSAACCCA3′ and reverse primer 5′YGAWACGGCTTCTCTCC3′ (annealing temperature 60°C); *Nanog* (transcription factor), forward primer 5′GTCTKCTRCTGAGATGC3′ and reverse primer 5′ASTKGTTTTTCTGCCACC3′ (55°C); *Oct-4*, forward primer 5′CTTCGGATTTCGCCTTCTCG3′ and reverse primer 5′CCTTGGAAGCTTAGCCAGGTC3′ (60°C), and PECAM/CD31 forward primer 5′AAGGTCAGCAGCATCGTG3′ and reverse primer 5′AGTGCAGATATACGTCCC3′ (56°C). Rex1 and Nanog primers were degenerate, so they could amplify mouse and human transcripts. Oct-4 primer sequences were kindly supplied by Dr. C. Verfaillie from the University of Leuven, Belgium. PCR conditions were: initial denaturation at 94°C for 3 min followed by 40 cycles at 94°C for 45 s, annealing for 45 s and 72°C for 45 s with a final extension at 72°C for 3 min. PCR products were separated by 1.5% agarose gel electrophoresis. DNA markers were used to confirm the size of the resultant fragments. To characterize differentiation of IDPSC, all primers were produced with established GenBank sequences. The following primers were used for amplification by RT-PCR: chondrogenic differentiation, AG, forward primer 5′GCAGAGACGCATCTAGAAATT3′ and reverse primer 3′GGTAATTGCAGGGAACATCAT5′; muscle, MyoD1(MD1), forward primer 5′AAGCGCCATCTCTTGAGGTA3′ and reverse primer 3′GCGCCTTTATTTTGATCACC5′; nerve, nestin, forward primer 5′GGAGTCGTTTCAGATGTGGG3′ and reverse primer 3′AGCTCTTCAGCCAGGTTGTC5′, and GFAP forward primer 5′AATGCTGGCTTCAAGGAGAC3′ and reverse primer 3′CCAGAGACTCAATCTTCCTC5′. Total human skeletal muscle and brain and primary human chondroblast RNA (Ambion, Austin, Tex., USA) were reverse transcribed and amplified by PCR as a positive control for lineage-specific differentiation, respectively. Undifferentiated IDPSC were examined as a negative control.

Engraftment of IDPSC

All animal procedures were performed in accordance with institutional guidelines. BALB/c nude mice (Biotério de Camundongos Isogênicos ICB/USP) received intra-peritoneal injection of 10^6 IDPSC. Animals (n = 10) were sacrificed 1, 2, and 3 months after injection by approved methods, and tissues of interest were freshly frozen to verify the presence of human cells. DNA extraction and nested PCR screening of human cells has been described elsewhere [Sambrook et al., 1989]. The following primers were used: 8a11F, 5′ACAAATTAGCTGGGTGTGGC3′; 8a11R, 5′TAAGCTCACACTGGCCCTGC3′; followed by A9F, 5′CCCAGTGTTGAGCCTTTGTC3′, and A9R, 5′AAGCCATCCGATGTAGGAGA3′ for amplification of human Goshe syndrome gene (GBA).

To analyze engraftment of IDPSC in murine tissues, polyclonal antibody was produced by immunizing Chinese hamsters (CH) with IDPSC according to standard protocols [Harlow and Lane, 1988]. Briefly, approximately 2.0×10^6 culture-expanded IDPSC without adjuvant were initially intraperitoneally injected into the CH. The initial injection was followed by four booster injections of such stem cells prepared in a similar fashion. After the 25th day, blood was drawn from the CH and the serum from the blood was assayed by indirect immunofluorescence using cultured IDPSC in order to check that the immunization regimen had been successful in generating an immune response in the CH to the cultured IDPSC. Cross-reactivity of secondary antibodies to murine tissue was minimized by using the antibodies absorbed with mouse serum. The serum was also assayed by indirect immunofluorescence using frozen sections of a variety of tissues that were obtained at surgery or autopsy from CH and mice. Different dilutions of the serum were screened in order to detect more appropriate concentrations.

From each organ, concurrently characterized by human GBA gene expression, serial cryosections (5–7 μm) were obtained which were immunostained with the obtained serum (dilution 1/1,000) followed by FITC-conjugated secondary antibody (dilution 1/500) according to the protocol described above. Nuclei were counterstained with propidium iodide (PI) and slides were mounted with antifade Vectashield (Vector, Hercules, Calif., USA).

Confocal Microscopy

An argon ion laser set at 488 nm for FITC and at 536 for PI excitation was used. The emitted light was filtered with a 505 nm (FITC) and 617 nm (PI) long pass filter in a laser scan microscope (LSM 410; Zeiss). Sections were taken approximately at the mid-height level of tissues. Photo-multiplier gain and laser power were kept constant throughout each experiment.

Results and Discussion

Dental pulp from exfoliated deciduous teeth of 5- to 7-year-old children (n = 10) was used in this study. The dental pulp retrieved from the tooth was mechanically dissected into small pieces to provide adequate medium exposure during initial dental pulp outgrowth culture. This culture (tissue explant) was maintained in DMEM/F12 medium supplemented with 15% FBS (HyClone) known to provide an optimal environment similar to those for ES cells [Laslett et al., 2003]. After 5 days of culture, we observed a robust exodus of fibroblast-like cells from these pieces of the dental pulp (fig. 1a). The same piece of dental pulp without trypsinization was transferred sequentially 15 times to a fresh Petri dish. Following each transfer, approximately 2×10^6 new cells in 10 days were observed to be covering the entire 35-mm dish (fig. 1a, inset).

After about 2 weeks of culture, the cells collected from the monolayer were washed twice in PBS, dissociated in a 0.25% trypsin solution, and seeded at 10^4 cells per 25-cm^2 flask. The culture was maintained semiconfluent and passed every 4–5 days, while the medium was replaced daily. The cell cultures established from all patients had high proliferation rates yielding approximately 3×10^7 cells within 2 weeks, as is expected for the expansion of primary cells. No changes in morphology or growth pattern were observed during 25 passages. The entire cell population was proliferatively active, as was assessed by flow cytometry with proliferating cell nuclear antigen antibody (fig. 1b, c). The cloning capacity of the cells on passage 10 was very high with 108 colonies per 110 plated cells. The growth kinetics of the single colony (n = 4) was measured at passage 25. For 16 days, cells were harvested and counted daily. No changes in growth rate were observed (fig. 1d), and all cells studied showed a normal karyotype (fig. 1e). Predictably, the entire population of ex vivo expanded undifferentiated cells was uniformly positive for AP (fig. 1f), which confirms their undifferentiated nature [Talbot et al., 1993]. Furthermore, we verified by RT-PCR the expression of osteonectin and osteocalcin in undifferentiated IDPSC and IDPSC with induced osteogenic differentiation. As expected [Zuk et al., 2002], we observed osteonectin expression in both, undifferentiated and induced osteogenic differentiation IDPSC, while osteocalcin expression was restricted to the time of osteogenic differentiation induction (data not shown).

Interestingly, despite the high density of the cells exiting the dental pulp, we observed neither differentiation nor slowed proliferation as could be expected for confluent cell cultures, particularly in the absence of specific growth factors. At the same time, both differentiation and slowed proliferation were noted during the subsequent confluent culture following trypsinization. This observation led us to believe that explanting dental pulp as an outgrowth culture before the initial passage with trypsinization may prevent any stem cells from undergo-

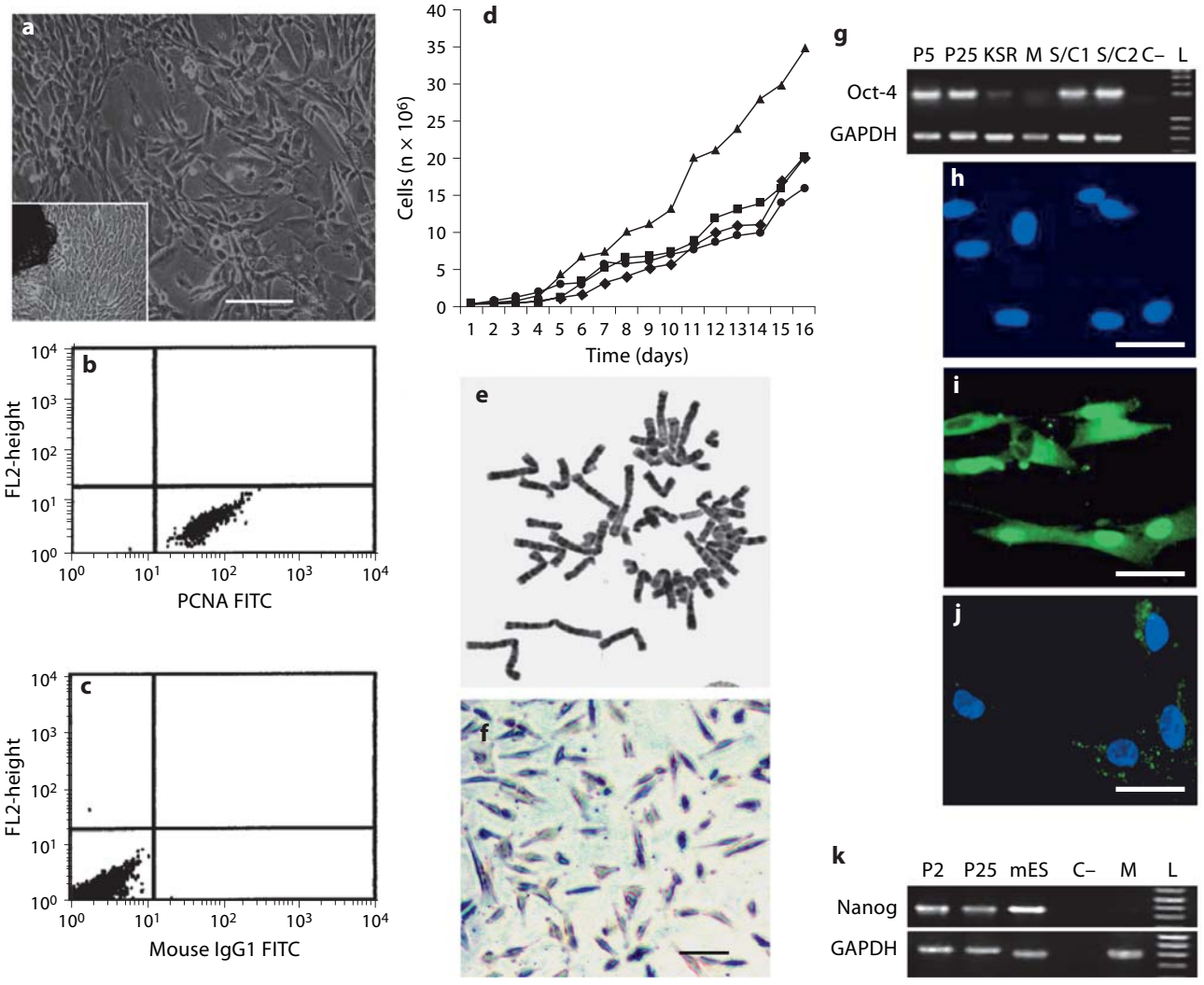

Fig. 1. Markers, growth pattern, and gene expression profile of IDPSC. **a** Culture of IDPSC. Inset: a piece of dental pulp from which IDPSC are migrating into the culture. **b** Nearly uniform expression of proliferating cell nuclear antigen by IDPSC is demonstrated by FACS analysis. **c** Negative control for **b**. **d** Growth kinetics of four representative IDPSC lines as a function of passage number. **e** IDPSC show normal karyotype (2n = 46, XY) at passage 25 (P25); G-banding, light microscope. **f** IDPSC are positive for AP activity at P25. **g** RT-PCR of Oct-4 expression in IDPSC. P5/P25 = IDPSC at P5/P25, with strong expression of Oct-4; KSR = Oct-4 expression is reduced 12 h following transfer into DMEM^{Knockout} medium supplemented with KSR; M = Oct-4 expression is not detectable after IDPSC differentiation into skeletal muscle; S/C1/S/C2 = strong Oct-4 expression in two IDPSC subclones at P4; C– = negative control; L = mass ladder. Bottom level = internal control for GAPDH. **h**, **i** IDPSC immunostaining for Oct-4. **h** The same field visualized through DAPI filter (blue) and **i** through FITC filter (green) for Oct-4 expression, respectively. Strong nuclear labeling with antibody to Oct-4 is seen in 6 out of 8 cells. Some cytoplasmic staining is also observed in all cells. **j** Negative control. No Oct-4 nuclear labeling is detectable in human primary fibroblasts. **h–j** Epifluorescence. **k** RT-PCR of Nanog expression. P2/P25 = IDPSC at P2 and P25; mES = mouse ES cells – positive control; C– = negative control; M = negative control, skeletal muscles differentiated from IDPSC; L = ladder. Scale bars: **a**, **f** 50 μm; **h–j** 20 μm.

Table 1. Marker expression profile of IDPSC, mMAPC and human ES cells

Markers	Cell type		
	human ES	mMAPC	IDPSC
Oct-4	+	+	+
SSEA-3	+	–	+
SSEA-4	+	–	+
TRA-1-60	+	–	+
TRA-1-81	+	–	+/–
Nanog	+	–	+
Rex-1	+	+	–
SH-2	n/a	n/a	+
SH-3	n/a	n/a	+
SH-4	n/a	n/a	+
CD13	n/a	+	+
CD34	n/a	n/a	–
CD43	n/a	n/a	–
CD45	n/a	n/a	–
CD31	n/a	–	+

+ = Positive marker expression; – = lack of marker expression; n/a = marker expression not analyzed.

ing premature differentiation. It is known that under normal tissue circumstances, most of the stem cells should be dormant and divide at a very slow rate [Cheshier et al., 1999]. However, when activated, for example by an injury, they begin to divide rapidly while migrating toward the injured site [Fleming et al., 1993]. It is likely that these cells represent a subpopulation of IDPSC that under our experimental conditions were predominantly and selectively proliferating. Furthermore, it would seem that the gradient between the piece of dental pulp and the culture medium served as a vector directing the cells toward what they perceive as a site of injury, which leads to their continued and selective migration in the Petri dish.

Unexpectedly, RT-PCR revealed expression of Oct-4 [Constantinescu, 2003; Laslett et al., 2003], which persists throughout passage 25 and is also observed in subclones expanded from a single cell (fig. 1g). Staining with antibodies confirmed that about 80% of the cells are strongly positive for Oct-4 (fig. 1h, i), while, as expected, human primary fibroblasts are negative for this marker (fig. 1j). The significance of Oct-4 expression in only a portion of the cells is unclear. It should be noted in this respect that only a small fraction of amniotic stem cells was shown to be positive for Oct-4 (5), while the percentage of Oct-4-positive cells varied from <0.01 to 80%, depending on the O_2 concentration in mMAPC [Jiang et al., 2002]. The lack of Oct-4 expression in some cells could be explained by contamination with non-stem cells. However, during normal development, Oct-4 is expressed in a mosaic pattern in the population of cells that otherwise have equal differentiation potential [Kirchhof et al., 2000]. Consistent with this observation, as shown below, we noted uniform differentiation of the entire stem cell population toward the same tissue type.

Furthermore, RT-PCR revealed that the outgrowth cells express Nanog, another marker of embryonic stem cells [Chambers, et al., 2003; Mitsui et al., 2003] (fig. 1k). As expected, Oct-4 and Nanog, markers of pluripotent cells, became downregulated following differentiation (fig. 1g, k). Other ES markers, SSEA-3, SSEA-4, and TRA-1-60, were also uniformly expressed in all presumably Oct-4-positive cells (fig. 2 a–c) but not in human primary fibroblasts (fig. 2d), while TRA-1-81 was expressed in only about 20% of the cells (data not shown). Expression of Rex-1, commonly used to confirm the presence of undifferentiated ES cells [Ward et al., 2003] and mMAPC, was not detected in these cultures. It should be noted that Rex-1 expression in mMAPCs under the O_2 concentrations used in this study was detectable in only 0.01 to 1% of the cells [Jiang et al., 2002]. This low level of expression could be below the threshold detectable by the methods used in the current experiments.

Additionally, the outgrowth cells were uniformly positive for human mesenchymal stem cell-specific antigens SH-2, SH-3, and SH-4 (fig. 2e–g), while human primary fibroblasts were negative for all these markers (fig. 2h). The uniformity of expression of CD105 (SH-2) was further confirmed by flow cytometry (fig. 2i, n). Furthermore, flow cytometry revealed that outgrowth cells were uniformly negative for CD34, CD43 and CD45 ruling out contamination with hematopoietic and endothelial cells (fig.1c, 2j–ln), while being positive for CD13 marker of hematopoietic precursors (fig. 1c, 2m). Thus, the outgrowth cells have an expression profile that partially overlaps with both human ES cells and mMAPCs (table 1). The difference in population purity between SHED and the population of cells described in the present study leads us to believe that they are not the same. Furthermore, we were able to isolate a population of identical cells from non-deciduous, permanent adult teeth [Kerkis, unpubl.], whereas SHED have only been described in deciduous teeth. Due to their apparent immature nature and persistence throughout childhood and adulthood, we named the outgrowth cell population IDPSC.

Stem cell differentiation into functional tissues remains a major challenge. Even though there are a number

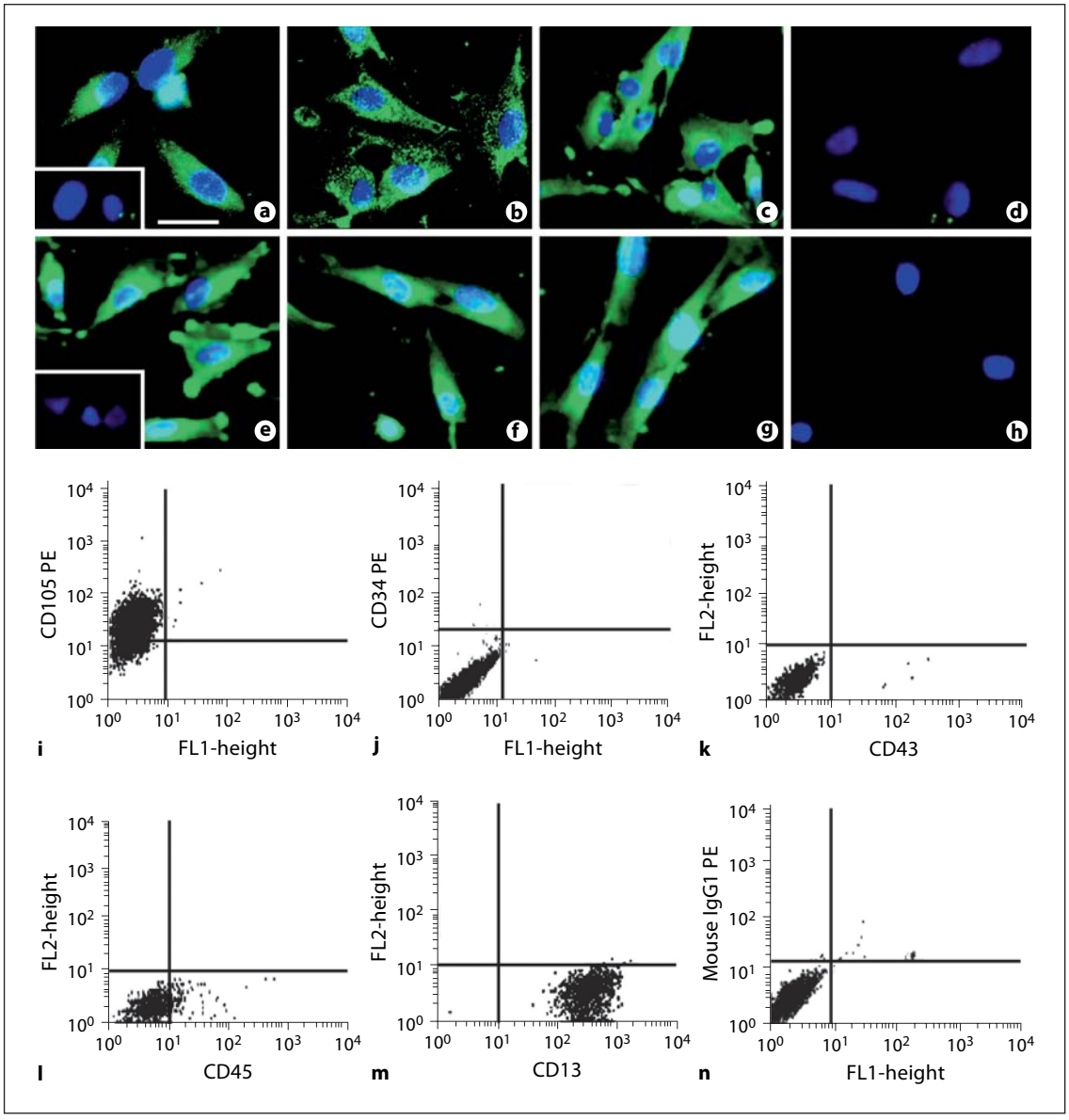

Fig. 2. Expression profile of IDPSC (continued). **a–c** IDPSC immunostaining for human ES cell markers. **a** SSEA-3. **b** SSEA-4. **c** TRA-1-60. **d** Negative control for TRA-1-60 with primary human fibroblasts. Inset in **a** shows respective isotype control for FITC-conjugated secondary antibody only. **e–g** IDPSC immunostaining for human mesenchymal stem cell markers. **e** SH-2. **f** SH-3. **g** SH-4. **h** Negative control for SH-4 with primary human fibroblasts. Inset in **e** is a respective isotype control for FITC. **a–h** Epifluorescence; scale bar = 20 μm. **i–n** IDPSC FACS analysis. IDPSC are positive for CD105 (**I**; SH2) and CD13 (**m**). IDPSC are negative for CD34 (**j**), CD43 (**k**) and CD45 (**l**). PE = Phycoerythrin. **n** Negative control (FITC) for **i** and **j**. Negative control for **k–m** is shown in figure 1c.

of described chemical inducers, like RA, which are able to direct stem cells into a desired phenotype in vitro, it is difficult to obtain uniformly induced differentiation without noting the presence of non-responsive cells [Rohwedel et al., 1999]. As an alternative to chemically induced differentiation, an apparently 'spontaneous' differentiation of stem cells triggered by cell confluence or the withdrawal of growth factors or substrates has been observed, and this is particularly common in ES cells [Reubinoff et al., 2000]. As a part of a preliminary study (data not shown), we observed that IDPSC could be directed toward desired phenotypes accompanied by a reduction

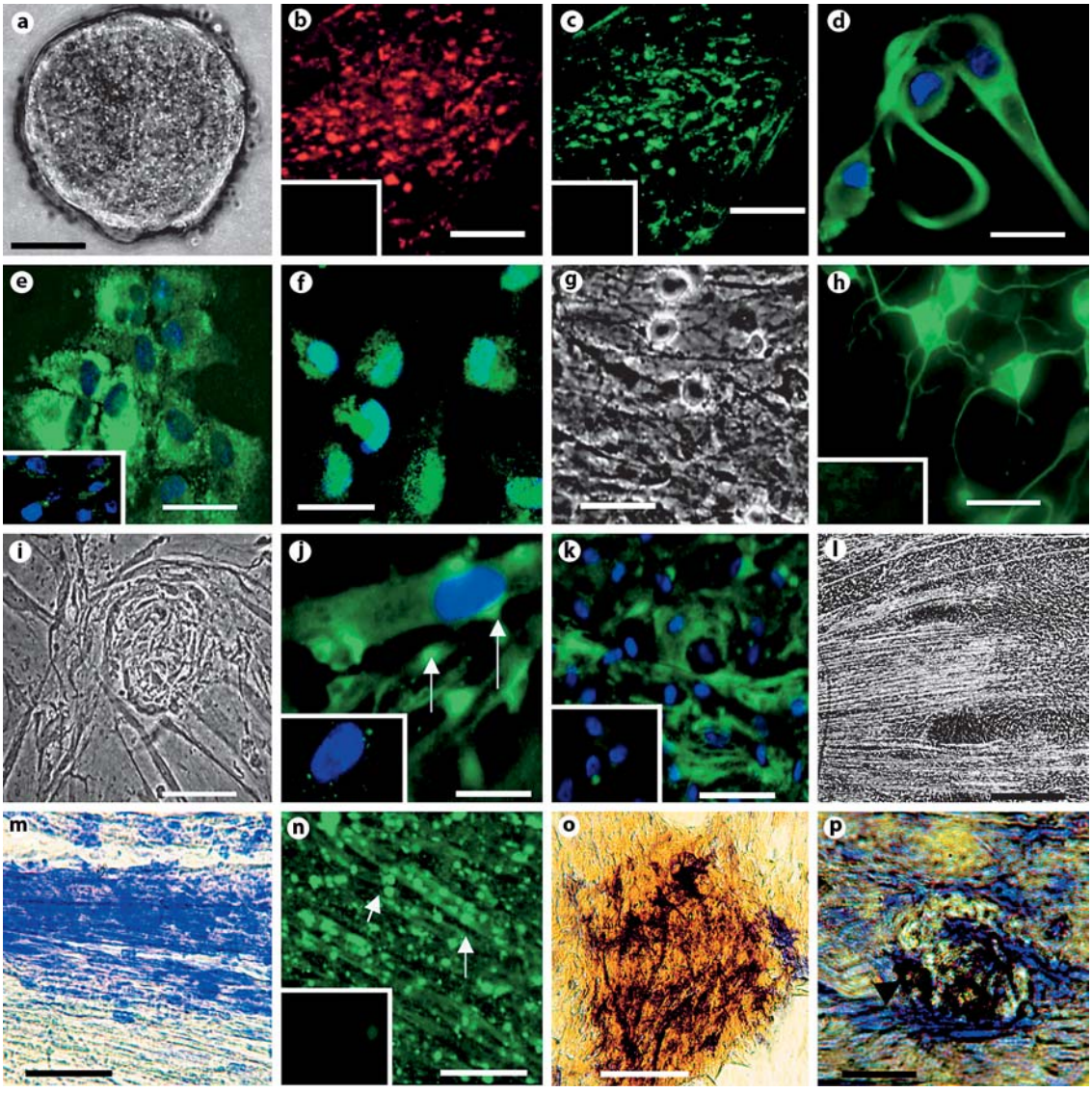

Fig. 3. IDPSC neuronal and chondrogenic differentiation. **a** The initial stage for both RA-induced and 'spontaneous' neuronal differentiation was via SLS formation. **b–d** RA induction of differentiation. **b** Adherent SLS express nestin (red). **c** β-Tubulin III (green). **d** IDPSC differentiated into motor neuron-like cells positive for β-tubulin (green). **d**, **e** DAPI-stained nuclei. **e–h** IDPSC 'spontaneous' neuronal differentiation that was also initiated in SLS as in **a**. **e** Nestin-positive (green) rosette structure. **f** GFAP-positive immunostaining of the nuclei (green) confirms glial nature of the cells. **g** Differentiation into neuron/glial sandwich resembling the primary culture of central nervous system. **h** β-Tubulin III-positive neurons from IDPSC (green). Insets show respective isotype controls for Cy3 (red) and FITC (green). **a**, **g** Phase contrast. **b–f**, **h** Epifluorescence. Scale bars: **a** 300 μm; **b**, **c**, **g** 50 μm; **d–f**, **h** 20 μm. **i–n** IDPSC chondrogenic differentiation. **i** First signs of chondrogenic differentiation can be seen 7 days after SLS adherence. **j** ACSP antibody-positive staining. Arrows show secretion of chondroitin sulfate proteoglycan granules (AG, green) from differentiating IDPSC. **k** Differentiated IDPSC, living culture. Anti-fetal cartilage proteoglycan antibody-positive immunostaining (green). **j**, **k** DAPI-stained nuclei (blue). **l–n** IDPSC highly differentiated cartilage (**l**) evidenced by metachromasia with toluidine blue staining (**m**) and positive immunostaining for AG granules (arrows, ACSP antibody) and a complete disappearance of nuclei (**n**). **o** IDPSC osteogenic differentiation. Positive AP staining observed 5 days after SLS adherence. **p** Bone-like structures (black arrow) stained by von Kossa silver method surrounded by toluidine blue counterstained for cartilage (metachromasia). Insets show respective isotype control for FITC. **i**, **l** = Phase contrast. **j**, **k**, **n** Epifluorescence. Scale bars: **i**, **l–p** 100 μm; **k** 50 μm; **j** 10 μm.

in Oct-4 expression (fig. 1g) by manipulating the seeding cell density and the time of the transfer into chemically defined medium: DMEM supplemented with KSR.

Relative to neuronal differentiation, the response of IDPSC to RA and their 'spontaneous' differentiation in KSR-supplemented medium were compared. For RA-induced differentiation, following trypsinization, the suspension of IDPSC was transferred into Neurobasal culture medium supplemented with B27 [Fraichard et al., 1995]. Within 24 h, the cells formed floating SLS (fig. 3a) at which point in time, neural differentiation was induced with 10^{-7} M RA and 0.05% dimethylsulfoxide. After 4 days of culture in non-adherent conditions with the medium changed daily, SLS were formed and attached to gelatin-coated dishes in Neurobasal + B27 culture medium. After 1 week in culture, about 60% of the cells expressed neuronal markers (fig. 3b, c), and about 40% of them had a distinctive appearance of neurons (fig. 3d). To promote 'spontaneous' neuronal differentiation, DMEM/F12 was replaced with DMEM/20% KSR without trypsinization when IDPSCs reached 90–100% of confluence. Cells remained in the same Petri dish and were maintained at confluence for 1 week. By the end of the week, cells formed a layer that partially resisted trypsinization. At that point, the cells were transferred to a new Petri dish, and within 24 h they formed SLS (fig. 3a) that attached to the bottom of the dish and transformed into nestin-positive rosette structures (fig. 3e); 3–4 days later these rosettes transformed into a uniform layer of well-distinguished glial cells overlaid with neurons (fig. 3f–h). The entire IDPSC population of differentiated cells was expressing neuronal markers (fig. 3h, 4). Every 3 days for 4 months, the glial cell layer was washed free of loosely attached neurons, which were sequentially transferred to a new Petri dish, while the cell layer continued to produce new neurons. This experiment demonstrated that 'spontaneous' differentiation of IDPSC takes place rapidly and was more uniform than was observed when chemically induced. Interestingly, both chemically induced and particularly 'spontaneous' differentiation took place about 4 times faster than neuronal differentiation previously described for SHED. By itself, the neuronal differentiation of IDPSC may not be surprising. However, uniform differentiation with formation of a glial/neuronal sandwich morphologically resembling primary neuronal cultures from the central nervous system has not been described previously for any adult precursors of non-neural origin, including SHED.

For chondrogenic differentiation, SLS from IDPSC were first obtained by the method described above for neuronal differentiation, however using culture medium for undifferentiated IDPSC (fig. 3a). Following this, SLS were plated between two coverslips. The first signs of differentiation were detected after 1 week of culture. Chondrogenesis appeared to expand from the initiation sites composed of undifferentiated cells that become enlarged due to the expression and accumulation of a proteoglycan-rich extracellular matrix (fig. 3i). After 2 weeks, cartilage formation was demonstrated with ACSP-AG (fig. 3j) and anti-fetal cartilage proteoglycan antibodies (fig. 3k) and toluidine blue dye staining (fig. 3l, m). AG expression by differentiated IDPSC was also shown by RT-PCR analysis (fig. 4). Notably, slides with highly differentiated cartilage mounted with DAPI/antifade did not show nuclear staining (fig. 3n). When SLS were adherent and cultured under the same conditions, but in the absence of the upper coverslip, we also observed osteogenic differentiation, as was confirmed by the presence of the osteo-specific AP activity and von Kossa staining (fig. 3o, p).

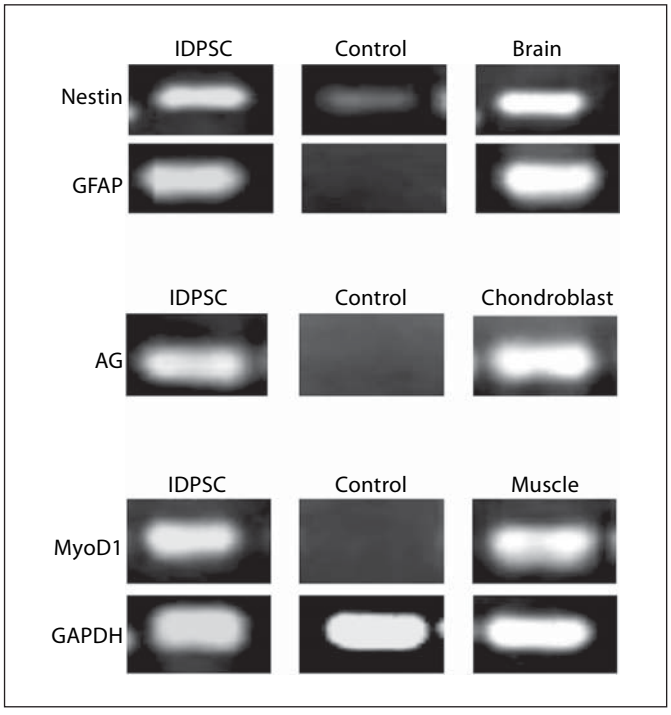

Fig. 4. Expression of lineage-specific genes by IDPSC during differentiation. IDPSC differentiation was analyzed by RT-PCR for the expression of lineage-specific genes: nestin, GFAP, AG, and MyoD1, respectively. Undifferentiated cells were used as a negative control. Total human skeletal muscle, brain and chondroblast RNA was used as a positive control. Lineage-specific gene expression was standardized in terms of GAPDH mRNA.

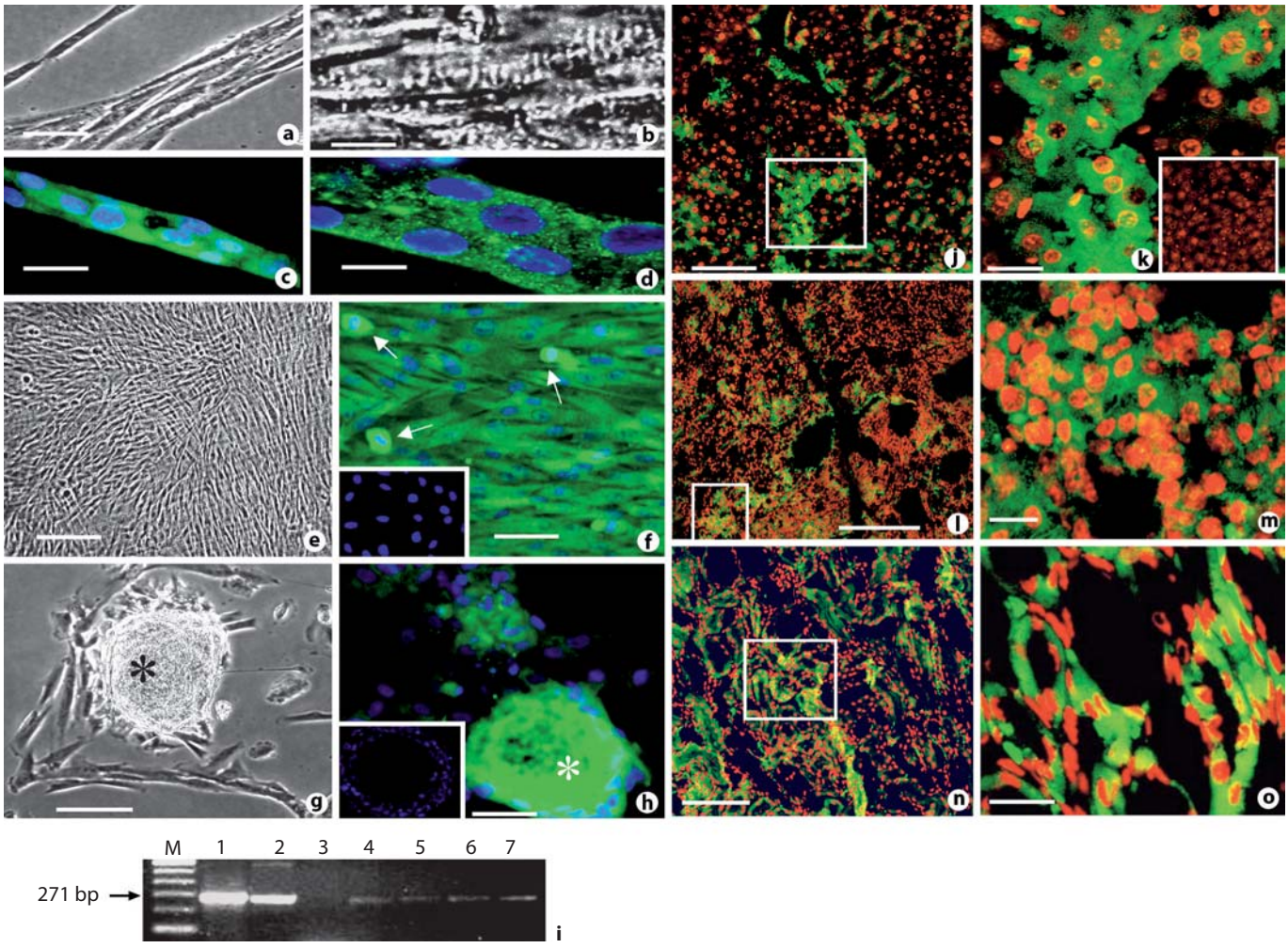

Fig. 5. Myogenic differentiation of IDPSC in vitro and their engraftment after in vivo transplantation into mice. **a–d** IDPSC differentiation into skeletal muscle. **a** Myoblast fusion. **b** Mature skeletal muscle fiber shows Z-disk. **c**, **d** Immunostaining with antibodies against titin (**c**) and against α-actinin (sarcomeric; **d**). **e–h** IDPSC differentiation into smooth muscle. **e** Living culture. **f** Positive immunostaining for smooth muscle actin. Uniform positive staining of the cytoplasm (green) is apparent. **g**, **h** Following confluence multicellular smooth muscle nodule formation from IDPSC (*). **h** Immunostaining for smooth muscle actin. **f**, **h** Insets show respective isotype controls for FITC. **c**, **d**, **f**, **h** DAPI-stained nuclei. **a**, **b**, **e**, **g** Phase contrast. **c**, **d**, **f**, **h** Epifluorescence. Scale bars: **e** 100 μm; **a**, **f–h** 50 μm; **b**, **c** 20 μm; **d** 10 μm. **i–o** Engraftment of IDPSC following intraperitoneal injection into nude mice (confocal microscopy). **i** Evidence of engraftment in different tissues of IDPSC recipients by PCR for human GBA gene 3 months after injection. M = 100-bp marker; 1 = positive control for human DNA; 2 = positive control for IDPSC DNA; 3 = negative control for mouse DNA; engraftment is detectable in 4 (lungs), 5 (liver), 6 (kidney) and 7 (brain). **j–o** Evidence of engraftment by staining with anti-IDPSC antibody, FITC-positive labeling (green), nuclei are counterstained with PI (red). Engraftment is evident in the liver (**j**, **k**), spleen (**l**, **m**) and kidneys (**n**, **o**). **k**, **m**, **o** Higher magnifications of the insets in **j**, **l**, **n**, respectively. Inset in **k** shows negative control for anti-IDPSC antibody using liver tissue from control mice (without IDPSC injection). Scale bars: **j**, **l**, **n** 100 μm; **k**, **m**, **o** 20 μm.

To promote skeletal muscle cells, IDPSCs were plated at a low density (10^3) in DMEM with 20% KSR. Within 1 day following seeding, the first myoblasts fused to form small myotubes (fig. 5a). These immature myotubes were thin and short with centrally located, clustered nuclei, which differentiated during the next 3 days into large multinucleate syncytia (data not shown). Approximately 6 days later, spontaneously contracting myofibers were formed. The sarcoplasm of most mature myotubes was filled with a large number of fully developed myofibrils

with cross-striated morphology and discernable Z-disks (fig. 5b). The process of myogenic differentiation was confirmed with antibodies against titin and α-actinin (sarcomeric; fig. 5c, d) and by RT-PCR analysis for MyoD1 gene expression using RT-PCR analysis (fig. 4). Titin was the first myofibrillar protein expressed after the induction of differentiation, even before cell fusion occurred.

In contrast to skeletal muscle, differentiation toward smooth muscle cells was induced by seeding cells at high density (~10^6). When allowed to remain as a monolayer, differentiating IDPSC did not exhibit contact inhibition even after confluence (fig. 5e), while the entire layer of cells gradually curled up and displayed a high resistance to trypsinization. The differentiated cells were positive for anti-smooth muscle actin by staining with a monoclonal antibody (fig. 5f). After confluence, the cells were capable of forming multicellular nodules and spheroids that expressed actin (fig. 5g, h) similar to previously described aortic and human placental pericytes [Dartsch et al., 1990; Schor et al., 1990].

The capacity of homogeneous differentiation under chemically defined conditions seems to be a hallmark of IDPSC. Interestingly, the pattern of seeding density required to promote differentiation of IDPSC in our study was similar to that required for differentiation of postnatal human marrow mesodermal progenitor cells [Reyes et al., 2001].

IDPSC were further characterized by their ability to engraft following intraperitoneal injection of BALB/c nude mice (n = 15) with 10^6 cells of each tested line (n = 4). When mice were sacrificed 3 months following IDPSC injection, human DNA sequence encoding the GBA gene was detected by PCR in different tissues (fig. 5i), and dense engraftment sites, morphologically indistinguishable from the host organs, were found in the liver, spleen, brain, kidney (fig. 5j–o), and other organs (data not shown).

Based on IDPSC differentiation plasticity we believe that they have a broad therapeutic potential, including treatment of neurotrauma, myocardial infarct, connective tissue damage and more.

The relationship between IDPSC, adult DPSC [Gronthos et al., 2002] and SHED [Miura et al., 2003] is an interesting question that has not been addressed in the present work and requires further investigation. However, the difference in population purity and differentiating potential between SHED and the population of cells described in the present study lead us to believe that they are not the same. Since IDPSC express markers of less mature stem cells, it is tempting to speculate that they may be multipotent precursors of both DPSC and SHED, similar to mMAPC isolated from bone marrow [Jiang et al., 2002].

In summary, our data suggest that non-exfoliated deciduous teeth are a reliable and abundant source of pluripotent stem cells that can be induced to uniform differentiation under chemically defined culture conditions with high efficiency.

Acknowledgments

The authors are indebted Dr. C. Verfaillie from the University of Leuven, Belgium, for providing Oct-4 primer sequences, Dr. R. Rosenberg from the Universidade de São Paulo, Brazil, for providing human GBA gene primer; Dr. R. Ribeiro dos Santos from the Fundação Fiocruz da Bahia, Brazil, for developing anti-IDPSC antibodies, and Mrs. Serrano for technical assistance in the FACS analysis. This work was supported by Clínica CERA, CNPq and FAPESPS.

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