Blastema cells derived from New Zealand white rabbit's pinna carry stemness properties as shown by differentiation into insulin producing, neural, and osteogenic lineages representing three embryonic germ layers

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Abstract Stem cells (SCs) are known as undifferentiated cells with self-renewal and differentiation capacities. Regeneration is a phenomenon that occurs in a limited number of animals after injury, during which blastema tissue is formed. It has been hypothesized that upon injury, the dedifferentiation of surrounding tissues leads into the appearance of cells with SC characteristics. In present study, stem-like cells (SLCs) were obtained from regenerating tissue of New Zealand white rabbit's pinna and their stemness properties were examined by their capacity to differentiate toward insulin producing cells (IPCs), as well as neural and osteogenic lineages. Differentiation was induced by culture of SLCs in defined medium, and cell fates were monitored by specific staining, RT-PCR and flow cytometry assays. Our results revealed that dithizone positive cells, which represent IPCs, and islet-like structures appeared 1 week after induction of SLCs, and this observation was confirmed by the elevated expression of *Ins*, *Pax6* and *Glut4* at mRNA level. Furthermore, SLCs were able to express neural markers as early as 1 week after retinoic acid

treatment. Finally, SLCs were able to differentiate into osteogenic lineage, as confirmed by Alizarin Red S staining and RT-PCR studies. In conclusion, SLCs, which could successfully differentiate into cells derived from all three germ layers, can be considered as a valuable model to study developmental biology and regenerative medicine.

Keywords Blastema · Differentiation capacity · Stem cells · Insulin producing cells · Neural lineage · Osteogenic lineage

Introduction

Stem cells (SCs) are cells with self-renewal (proliferation) and differentiation potentials that are responsible for life long maintenance of cellular compartments, specially in adult tissues (Watt and Hogan 2000). SCs are classified into two main groups depending on their origin: embryonic SCs (ESCs) and adult SCs (ASCs) (Bissels et al. 2013). ESCs are pluripotent cells, derived from the inner cell mass of blastocyst-stage embryos (Evans and Kaufman 1981; Martin 1981) that under appropriate conditions will differentiate into cells of all three germ layers including ectoderm, mesoderm, and endoderm (Vazin and Freed 2010). ASCs, which are found in many adult tissues, have multi- or unipotent differentiation potential and are responsible for the maintenance and regeneration of tissues in which they

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are located (Ehnert et al. 2009; Gonzalez and Bernard 2012).

Despite their great differentiation potential, application of ESCs at clinical level is still facing a number of problems, including ethical concerns and legislative debates (Rosner et al. 2014; Teng et al. 2014). Furthermore, using ESCs in therapeutic procedures may result to tumorigenesis (Dressel et al. 2008; Ben-David and Benvenisty 2011). Accordingly, many laboratories have focused on derivation and therapeutic use of other sources of SCs, such as ASCs, which can be used in autologous therapies (Dantuma et al. 2010). Nevertheless, ASCs have a lower differentiation capacity, which limits their efficacy for clinical applications (Poulsom et al. 2002; Patel and Genovese 2011). These drawbacks have motivated scientists to look for cells involved in the regenerative phenomenon, which is a sequential set of cellular processes including dedifferentiation, migration, proliferation, and finally redifferentiation of cells close to the site of injury (Odelberg 2005; Carlson 2007). Cellular dedifferentiation is a unique process in which fully differentiated cells revert to proliferating progenitor cells that, in some cases, leads to the formation of a blastema tissue (Tsonis 2000; Brockes and Kumar 2002; Odelberg 2004; Stocum 2012). Therefore, blastema tissue contains undifferentiated and proliferating cells that are able to redifferentiate to specific cell types as required (Lemischka 1999; Carlson 2007). These processes, cellular dedifferentiation and blastema formation, are especially prominent in some animals with regenerative abilities, such as hydra (Galliot et al. 2006; Bosch 2007a, b), planarians (Reddien and Sánchez Alvarado 2004), zebrafish (Poss et al. 2003; Poss 2007), salamanders, axolotls and newts (Odelberg 2005; Brockes and Kumar 2005). There are also several reports on limited regeneration in mammals, including the regrowth of fingertips in mouse (Han et al. 2008), antlers in deer (Li et al. 2014), and ear hole closure in rabbit (Goss and Grimes 1975). The latter is one of the best examples of regeneration process in mammals, in which blastema tissue is formed on the periphery of the wound site (William-Boyce and Daniel 1980).

The objective of present study was to first derive stem-like cells (SLCs) from rabbit's pinna regenerating tissue, and then examine their differentiation potential, as an important stemness trait. To do so, SLCs were induced toward insulin producing cells (IPCs), neural cells and osteocytes in vitro.

Materials and methods

Animals

Three to 6 month-old male New Zealand white rabbits (*Oryctolagus cuniculus*) were obtained from Razi Vaccine and Serum Research Institute (Mashhad, Iran) and treated according to Ferdowsi University of Mashhad animal ethics directions. Rabbits were kept under standard conditions; controlled temperature (20–22 °C), a 12 h light/dark cycle and free access to water and standard rabbit chow (Javaneh Khorassan, Iran).

Isolation and culture of SLCs from rabbit's pinnas

Cell isolation was performed as previously described (Mahmoudi et al. 2011). Briefly, rabbit's ears were shaved and cleaned with 70 % ethanol and first punches were made in areas between the medial ear artery and the marginal ear veins, to make holes with 2 mm diameter in the pinnas. Two days later, second punches (4 mm in diameter) were made and O-shaped rings were obtained. Rings were then washed with physiological serum and culture medium, containing low glucose Dulbecco's modified Eagle's medium (DMEM; Gibco, Paisley, Scotland) supplemented with 15 % Fetal Bovine Serum (FBS; Gibco) and 1 % penicillin and streptomycin (Biosera, Uckfield, UK), and transferred into six-well plates containing the same medium. After 7–10 days culture at 37 °C in a humidified atmosphere containing 5 % CO₂, a homogeneous population of spindle-like cells from the rings started to attach to the surface of the plates. These cells, named as SLCs, were subcultured after reaching confluency using 0.25 % trypsin–1 mM EDTA (Gibco) and transferred into 25-cm² culture flasks. The culture medium was refreshed every 2 days and cells were passaged twice a week at a ratio of 1:2.

Cultivation of positive control cell lines

NTERA-2 cells and bone marrow derived Mesenchymal SCs (MSCs) were considered as positive controls in present study. NTERA-2 cells, generously provided by Prof. Peter Andrews (University of Sheffield, UK) were cultured in high glucose DMEM (Gibco) supplemented with 10 % FBS. Moreover, rat MSCs (rMSCs) were extracted from femurs and tibias of 2-month old male Wistar rats, obtained from Razi

Vaccine and Serum Institute (Mashhad, Iran), as previously described (Neshati et al. 2010). rMSCs were cultured in low glucose DMEM containing 15 % FBS and 1 % penicillin/streptomycin. After removing floating cells and exchanging the medium, attached cells were subcultured and following three passages, rMSCs became morphologically homogeneous with a spindle-like appearance. Both cell lines were incubated at 37 °C in a humidified atmosphere of 10 % CO₂ in air, and subcultured, when required, with glass beads (Corning Life Sciences, Tewksbury, MA, USA) or 0.25 % trypsin–1 mM EDTA, respectively.

Induction of differentiation

Differentiation into IPCs

To induce differentiation, SLCs at passage 5, were plated at a density of 3.5×10^5 cells/well in 6-well plates. After 24 h and reaching 75–85 % confluency, SLCs were induced to differentiate into IPCs by a two-stage protocol, as previously described (Neshati et al. 2010). At first stage, cells were cultured in a differentiation medium containing serum-free high glucose DMEM (25 mM), 10 mM nicotinamide (Sigma, Munich, Germany), 0.5 mM β -mercaptoethanol (Invitrogen, Darmstadt, Germany) and 1 % penicillin/streptomycin for 2 days. In the second stage, cells were re-induced with 10 mM nicotinamide in serum-free high glucose medium for 26 days, while the culture medium was changed every 2 days. During the culture and differentiation period, morphology of cells was examined under an inverted light microscope (Olympus, Tokyo, Japan) connected to camera.

Dithizone staining

For dithizone (DTZ) staining, 10 mg DTZ (Sigma) was completely dissolved in 1 ml dimethylsulfoxide (DMSO, Merck, Darmstadt, Germany) and stored briefly at –20 °C. To stain the cells, 10 μ l of dissolved DTZ was added to 1 ml culture medium and cells were then incubated at 37 °C for 15–20 min. Afterwards, plates were rinsed with Phosphate Buffered Saline (PBS) for three times and observed under an inverted light microscope. After examination and photography, plates were refilled with serum-free high glucose medium containing 10 mM nicotinamide.

Insulin secretion from differentiated SLCs

To measure the concentration of extracellular insulin, culture media of induced and untreated SLCs were collected and assessed on days 2, 4, 6, 14, 21 and 28 after treatments. Four days prior to each time point, the medium was changed, except for the first one in which the medium was collected 2 days after induction. Insulin levels were measured by an immunoradiometric assay (INS-IRMA kit, Biosource, Belgium) according to the manufacturer's instructions. Briefly, the media were incubated with two monoclonal anti-insulin antibodies, one adsorbed on the tube walls and another one labelled by 125-I, at room temperature for 2 h. After several washings, radioactivity of precipitates was counted by Genesis γ -counter (Block Scientific, Bohemia, NY, USA).

To further study whether induced cells were responsive to glucose stimulation, IPCs were first incubated with medium containing 25 mM glucose at 37 °C for 2 days. Then, cells were exposed to media containing five different concentrations of glucose (5.5, 10, 15, 25 and 35 mM) for 4 days. Conditioned media were then collected and insulin concentrations were examined by immunoradiometric assay as described.

Reverse transcription polymerase chain reaction (RT-PCR) analysis

To determine the level of *Ins*, *Pax6* and *Glut4* expression, RT-PCR analysis was performed. To do so, total RNAs from induced SLCs (6 days after induction), non-induced SLCs and rat pancreas cell lysate (as positive control) were extracted using TRIzol Reagent (Ambion, Foster City, CA, USA), and single stranded cDNAs were synthesized using Moloney Murine Leukemia Virus (MMuLV) reverse transcriptase (Fermentas, St. Leon-Rot, Germany), according to the manufacturer's instructions. Then, PCR was performed using *Ins*, *Pax6* and *Glut4* primers (Table 1), which could amplify both rabbit and rat sequences. To note, the test cDNAs were normalized with controls using β -actin primers.

Differentiation into neural cells

SLCs were cultured and treated with 0.5×10^{-5} and 1×10^{-5} M retinoic acid (RA, Sigma) to induce

Table 1 Gene specific primers used for RT-PCR

Gene name	Primers	Product size (bp)	Accession number
<i>β-actin</i>	F: AAGATGACCCAGATCATGT R: AGGTCCAGACGCAGGATG	188	NM_001101683.1
<i>Ins</i>	F: TCCTGCCCCCTGCTGGC R: AGTTGCAGTAGTTCTCCAG	313	NM_001082335
<i>Pax6</i>	F: CATGCAGAACAGTCACAGCGG R: CCCATCTGTTGCTTTTCGCTA	456	NM_001082217
<i>Glut4</i>	F: CATGTGTGGCTGTGCCATC R: TGGCTGAAGAGCTCGGCC	162	NM_001089313.1
<i>Ocn</i>	F: CCTCGCTGGCCAGGCAG R: CTCCAGGGGATCCGGGTA	157	XM_002715383.2
<i>Opn</i>	F: CCTGACCCATCTCAGAAGC R: TCCACGTGGTCATGGCTTTC	122	NM_001082194.1

neural differentiation. In this experiment, NTERA-2 cells were considered as a positive control. One, two and three weeks after treatments, morphological alterations were observed by an inverted light microscope, and expression of neural markers (GT3 and GD3 gangliosides) was examined by flow cytometry. To do so, SLCs and NTERA-2 cells were harvested with 0.25 % trypsin–1 mM EDTA, washed three times with PBS containing 5 % FBS (washing buffer) and incubated with 1:50 diluted primary antibodies of A2B5 (anti-ganglioside GT3) and VIN-IS-56 (anti-ganglioside GD3), generous gifts from Prof. Peter Andrews (University of Sheffield, UK), for 45 min at 4 °C. Then, cells were washed three times and incubated with corresponded FITC-conjugated goat IgM (1:80 diluted, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for another 45 min at 4 °C in the dark. In the end, unbound antibodies were removed by washing and cells were resuspended in washing buffer and analyzed on FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA) using fl2 channel.

Osteogenic differentiation of SLCs

For osteogenic differentiation, SLCs at passage 6 and rMSCs at passage 4 (used as a positive control) were treated with DMEM containing 15 % FBS, 100 nM dexamethasone, 50 µg/ml ascorbate-2-phosphate and 10 mM β-glycerol phosphate (Sigma), as previously described (Ahmadian Kia et al. 2011). To note, osteogenic induction medium was replaced twice a week for up to 3 weeks.

Alizarin Red S staining and RT-PCR analysis

On day 20, induced SLCs and rMSCs were first fixed with 10 % formalin (Merck) for 30–40 min, and then stained with Alizarin Red S (Sigma) for 15 min at room temperature, so that the mineralized matrix of bone could be visualized. Furthermore, to determine the expression of Osteocalcin (*Ocn*) and Osteopontin (*Opn*) in induced cells, RT-PCR analysis was performed using specific primers (Table 1).

Results

Differentiation into IPCs

In the present study, differentiation of SLCs toward IPCs was induced at passage 5. Morphological observations revealed that unlike undifferentiated SLCs, which were adherent spindle-like cells (Fig. 1a), induced cells first became rounded (Fig. 1b) and then formed clusters quite similar to islet-like structures of the pancreas (Fig. 1c). To detect the insulin produced by islet-like structures, DTZ staining, which specifically detects insulin granules present in β-cells, (Shiroy et al. 2002; Chen et al. 2004) was performed. While no staining was observed in undifferentiated SLCs (control cells) (Fig. 2a), DTZ-positive cells were detected in differentiated cultures as early as 2 days after induction. Differentiated cells were also stained with DTZ on days 4, 7, 14, 21 and 28 after

Fig. 1 Morphological changes of SLCs during differentiation. The micrographs represent morphology of undifferentiated SLCs (a), and 4 (b) and 7 (c) days after induction of differentiation toward IPCs (scale bar 50 µm)

induction, while they presented grape-like and crimson red clusters, similar to pancreatic β -cells (Fig. 2d–h).

Immunoradiometric assay (IRMA) revealed that insulin concentration was 1.5 ± 0.2 μ IU/ml in the control medium, while 2, 4, 14, 21 and 28 days after induction, it increased up to 13.4, 13.9, 14.8, 12.9 and 12.3 μ IU/ml, respectively. Interestingly, the highest level of insulin was detected 6 days after induction (16.8 ± 1.2 μ IU/ml). Insulin secretion was also measured by IRMA after glucose stimulation. Results revealed that IPCs were not responsive to glucose concentrations, suggesting that induced cells probably represent immature β -like cells.

Further characterization of IPCs by RT-PCR indicated the expression of *Ins*, *Pax6* and *Glut4* in cells 6 days after induction, while no expression was detected in undifferentiated SLCs (Fig. 3).

Differentiation into neural cells

Alterations in growth and morphology were observed in SLCs, 4–7 days after RA treatment (Fig. 4). Interestingly, untreated SLCs expressed GT3 and GD3, and these neural markers were detected at higher levels on days 7 and 14 after induction. Our results indicated that the highest percentage of neural cells was detected on day 7, and longer treatments did not yield to greater percentages of positive cells. However, the highest expression of neural markers in NTERA-2 cells was detected on day 14 (Fig. 5).

Differentiation into osteocytes

Both SLCs and rMSCs presented osteogenic morphology 7 days after differentiation. Moreover, Alizarin Red S staining revealed deposition of mineralized matrix in both cell types 3 weeks after induction. To note, no osteogenic differentiation was observed in untreated cells (Fig. 6). RT-PCR analysis also indicated the expression of *Ocn* and *Opn* in cells 3 weeks after induction, while no expression was detected in undifferentiated SLCs (Fig. 7).

Discussion

Regeneration, a unique phenomenon that occurs in few animals, consists of several steps that are wound epidermis formation, blastema formation and redifferentiation (Bryant et al. 2002; Suzuki et al. 2006; Yokoyama 2008). Beside several reports on amphibian limb regeneration, ear hole closure in rabbit pinnae is known as one of the best examples of regeneration. However, there is not enough evidence to determine the exact origin of blastema in rabbit ear, as it forms from a population of SCs or by dedifferentiation of dermal fibroblasts (Stocum 2012). In this regard, we have previously reported that SLCs, obtained from the New Zealand white rabbit's pinnae, showed SC characteristics such as high proliferation ability and expression of pluripotency markers *Oct4* and *Sox2* (Mahmoudi et al. 2011). In the present study, the capacity of SLCs to differentiate into cells derived

Fig. 2 DTZ staining of SLCs during their differentiation toward IPCs. Undifferentiated SLCs presented spindle-like morphology with no DTZ development (a). DTZ-stained cells were visible on days 2 (b) and 4 (c) after induction. Cells made clusters on days 7 (d), 14 (e), 21 (f) and 28 (g) after induction. Islet-like *grape-shaped* cells isolated from induction medium presented similar structures to islets of Langerhans (h). Scale bars represent 50 μ m (a–c) and 20 μ m (d–h) in length

from all three embryonic germ layers was investigated. Our results indicated that SLCs were successfully induced into IPCs (as endodermal cells), neural cells (as ectodermal cells) and osteocytes (as mesodermal cells) under certain culture conditions.

It has been shown that serum-free medium (Zalzman et al. 2003) and β -mercaptoethanol (Kojima et al. 2004) promote the expression of *Nestin*, a pancreatic precursor marker, in SCs. Several studies have also indicated that high glucose concentration (25 mM) facilitates differentiation of SCs toward pancreatic

Fig. 3 RT-PCR analysis of *Ins*, *Pax6* and *Glut4* expression 6 days after induction. Numbers on top are described as follows, *lane 1*: induced SLCs; *lane 2*: rat pancreatic cells (positive control); and *lane 3*: non-induced SLCs. The *bottom row* represents β -actin expression which was used for cDNA normalization

lineages (Sun et al. 2007; Neshati et al. 2010). Moreover, it has also been demonstrated that combination of β -mercaptoethanol with serum-free medium or nicotinamide increases the efficiency of differentiation to IPCs (Kojima et al. 2004; Sun et al. 2007; Neshati et al. 2010). Based on above mentioned reports, we induced the differentiation of SLCs using a combined protocol, which includes nicotinamide and β -mercaptoethanol in a high glucose serum-free medium. Results revealed that induced cells shared similar morphology with pancreatic islet cells and produced crimson red cellular clusters after DTZ staining. Interestingly, DTZ-stained clusters were observed in differentiated SLC cultures only 1 week after induction, while our previous results indicated

that such structures were formed in differentiated rMSC cultures 2 weeks after induction (Neshati et al. 2010). Although induced SLCs were able to produce and secrete insulin, they did not respond to glucose stimulation. This was in line with previous reports indicating that induced ESCs might present immature β -like cells and more induction is required to reach a higher degree of differentiation (Gao et al. 2008; Kroon et al. 2008; Rezanian et al. 2012; Schulz et al. 2012). To further study differentiated SLCs, expression of *Ins*, *Pax6* and *Glut4* was assayed by RT-PCR. Results revealed that unlike undifferentiated SLCs, induced cells were able to express *Ins*, *Pax6* and *Glut4* similar to pancreas tissue.

To induce neural differentiation, SLCs were treated with RA, which has been introduced as a neural inducer in mouse and human embryonal carcinoma cells (Andrews et al. 1984; McBurney et al. 1988; Staines et al. 1994) and mouse ESCs in vitro (Rohweddel et al. 1999; Pacherník et al. 2005). Present results indicated that RA could induce neural differentiation in SLCs 7 days after treatment, as confirmed by detection of GT3 and GD3 neural markers. However, NTERA-2 cells, which are known as an excellent model for neural differentiation, were induced to neural lineage 14 days after RA treatment. Furthermore, according to our observations, the untreated SLCs expressed neural markers and could differentiate into neural lineage after a shorter induction period as compared with NTERA-2 cells.

Fig. 4 Morphological changes of SLCs after RA treatment. Induced SLCs showed a bipolar morphology 1 week after induction (a), while astrocyte-like cells were visible 3 weeks (b) after RA treatment. Scale bars represent 50 μ m (a) and 20 μ m (b) in length

Fig. 5 Flow cytometric analysis of neural markers in NTERA-2 and SLCs during RA treatment. Histograms present expression of A2B5 and VIN-IS-56 in cells treated with 0.5×10^{-5} and 10^{-5} MRA, during three continuous weeks. To note, the highest expression of neural markers was observed in treated SLCs during the first week

To investigate mesodermal differentiation of SCLs, an osteogenic medium, containing dexamethasone, ascorbate-2-phosphate and β -glycerol phosphate, was used based on several reports and our previous experience (Covas et al. 2003; Ahmadian Kia et al. 2011; Sun and Ji 2012). Differentiation of SLCs toward osteocytes was confirmed by their mineralized matrix stained with Alizarin Red S. RT-PCR studies also indicated the expression of *Ocn* and *Opn* in differentiated SLCs, quite similar to induced rMSCs.

Another study on SLCs also reported differentiation of these cells toward mesodermal lineages, including cartilage, bone, and adipose cells (Baghaban Eslaminejad and Bordbar 2013). However, in the current study, we demonstrated for the first time the successful differentiation of SLCs into IPCs, neural cells and osteocytes, representatives of all three germ layers.

Limitations and challenges of ESCs, such as their ethical concerns and tumor developing potentials, has made it crucial to find new cell sources for disease modeling and clinical therapy. Accordingly, discovery of multipotent SCs throughout the

Fig. 7 RT-PCR analysis of *Ocn* and *Opn* expression upon osteogenic differentiation. Numbers on top are described as follows, *lane 1*: induced SLCs; *lane 2*: induced rMSCs (positive control); *lane 3*: non-induced SLCs; and *lane 4* non-induced rMSCs. *Bottom row* represents β -actin expression that was used for cDNA normalization

human body not only indicated regeneration ability of adult tissues, but also opened new avenues in designation of cell-based strategies. The candidate cell sources suitable for therapeutic applications must possess several traits including easy accessibility and isolation, high proliferation and differentiation capacities, long cryopreservation and ideally no immunogenicity (Edwards 2008). According to the present results, which indicated differentiation of SLCs into endodermal, ectodermal and mesodermal lineages, SLCs could be considered as an accessible and remarkable cell source for future SC research. More studies, however, are needed to completely characterize SLCs and examine their tumorigenicity in vivo. Further research in this field might also give new insights in regeneration medicine and/or lead us to improve wound regeneration in patients.

Fig. 6 Alizarin Red S staining after osteogenic differentiation. rMSCs (a) and SLCs (b) were treated with osteogenic medium for 3 weeks. *Scale bars* represent 50 μ m in length

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