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# Blastema cells derived from rabbit ear show stem cell characteristics

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## Abstract

Regeneration is a biological phenomenon, which takes place via two main mechanisms: first, dedifferentiation of mature cells followed by their differentiation into functional new cells and second, activation of endogenous somatic stem cells for regeneration of damaged or lost tissues. One of the best examples of healing process in mammals is the regeneration of damaged pinna in rabbits by blastema tissue. The aim of present study was to investigate culture requirements, proliferative properties and expression of some stemness factors in cells derived from regenerating blastema tissue obtained from rabbit pinna *in vitro*. The regenerating tissues were obtained from male New Zealand white rabbits by double punching of the pinna and cell culture conditions were set to derive and enrich the self renewing cells for further characterisation. The cells were subjected to survival and growth examinations *in vitro*, and expression of several stemness factors was studied in these cells using reverse transcription polymerase chain reaction (RT-PCR). Results revealed that the derived cells are rather immortal, as they have been growing for more than 120 passages in culture up until this report. Furthermore, RT-PCR and flow cytometry analyses showed that these cells express a number of stemness related genes including *Oct4* and *Sox2*. In conclusion, in this study, stem like cells were derived from blastema tissue of rabbit ears for the first time, showing great self renewing capacity, which provides a suitable *in vitro* model for regeneration studies. Moreover, they could be considered as a good source of stem like cells for future applications.

***Keywords*:** regeneration, blastema tissue, pluripotency, stem cell

## Introduction

Derivation and applications of stem cells in recent years has taken the ground in many directions, including human health, as one of the most potential emerging fields. Since first radical report of successful derivation of human embryonic stem cells (ESCs) (Thomson et al., 1998), intense efforts have been dedicated to derive cells suitable for clinical applications. Due to several drawbacks, including ethical concerns and high risk of tumour development, ESCs are considered as low potential for therapiutic purposes (Stocum, 2002).

Meanwhile, several alternatives are being considered in parallel to cover the high expectations in regenerative medicine from stem cells. Among these, induction of dedifferentiation in somatic cells, and derivation and amplification of endogenous adult stem cells are of special interest for their potentials in regenerative medicine.

Several methods have been introduced for induction of dedifferentiation in differentiated cells to reach the cells with stemness features, including somatic cell nuclear transfer (SCNT) (Wilmut et al., 1997), induction of reprogramming in somatic cells by the fusion of cells with ES cells (Do and Scholer, 2004), and treatment of differentiated cells with extracts of pluripotent cells (Freberg et al., 2007; Hansis et al., 2004; Taranger et al., 2005). However, these methods proved to be too inefficient to be used in therapeutical programs. In 2006, direct reprogramming of somatic cells towards pluripotent state, by using defined transcription factors, was reported by Takahashi and Yamanaka (Takahashi and Yamanaka, 2006). In this approach, they transduced mouse embryonic fibroblasts (MEFs) with four transcription factors (*Oct4*, *Sox2*, *Klf4* and *c-Myc*). One of the main drawbacks of induced Pluripotent Stem (iPS) cell

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technology is employment of exogenous genetic factors (viral based constructs) for induction of reprogramming. In addition, some of these reprogramming factors (c-Myc and Klf4) have an oncogenic nature.

Regeneration is usually defined as reformation of body parts that are lost by injury. This phenomenon can occur in various styles, including production of dense collagen scar or through a scar free approach which is called epimorphic regeneration (Stocum, 2006). In invertebrate species, regeneration of lost parts is widely observed, while most vertebrates do not have such a remarkable ability (Brockes and Kumar, 2005; Endo et al., 2007; Masaki and Ide, 2007; Mochii et al., 2007). Among vertebrate species, urodeles and teleosts retain high ability for regeneration and are able to replace lost appendages (Poss et al., 2003).

Nearly all animals possess the capability of regeneration, but relatively only few species have ability of epimorphic regeneration. Our current knowledge about this phenomenon is largely gained from studies on lower vertebrates, particularly on amphibians (Brockes and Kumar, 2002), Nevertheless, some few mammals have also shown this ability. Rabbit ears are known to be capable of regenerating tissues producing by punching their pinnas (Dyson and Joseph, 1971; Goss and Grimes, 1972; Grimes and Goss, 1970). All other mammalian ears are incapable of regenerating full thickness. Instead, their inner and outer epidermis simply heal arround the margins and scar is formed, whereas, in the rabbits a circular blastema tissue is developed. Studies have demonstrated that regenerating rabbit ear tissues and cells have fascinating properties (Mahdavi-Shahri et al., 2008).

The ultimate goal of regenerating studies is to understand how lost or damaged cells are replaced and which mechanisms are involved in this phenomenon. Various mechanisms have been observed to be associated with regeneration in animals including, dedifferentiation, transdifferentiation and activation of somatic stem cells. For example, in zebrafish dedifferentiation and proliferation of cadiomyocytes has been reported to be involved in regeneration of the missing tissue of heart (Jopling et al., 2010; Kikuchi et al., 2010), or transdifferentiation was first observed in the regenerating lens of newts (Wolff, 1895), and activation of somatic stem cells located in a niche has been reported as the mechanism involved in regeneration (as occurs with blood) (Jopling et al., 2011).

Since one of the mechanisms associated with regeneration is dedifferentiation, studying this phenomenon in some animals can be useful as a simple system for decoding and promoting reprogramming technology.

In present study, stem like cells (SLCs) were derived from rabbit pinnas for the first time, and their morphology, growth rate and viability were investigated *in vitro*. Also, expression of genes such as *Oct4* and *Sox2*, which are characteristics of pluripotent cells was profiled.

## Materials and Methods

### *Animals*

3-6 month old male New Zealand white rabbits (*Oryctolagus cuniculus*), were purchased from Razi Vaccine and Serum Research Institute (RVSRI) (Mashhad, Iran). Rabbits were kept under standard conditions, fed with standard rabbit chow (Javaneh Khorassan, Iran) and tap water.

### *Preparing and culture of regenerating tissues*

Rabbit pinnas were shaved, cleaned with 70% ethanol and punched to make holes (2 mm in diameter). Punches were made in areas between the medial ear artery and the marginal ear veins, where there are few major vessels. Two days after first punching, the second punches (4 mm in diameter) were made and O-shaped rings were obtained. The rings were washed with physiological serum and culture medium for several times, and transferred into the six-well plates for culture.

### *Culture and collection of outgrowth cells from the regenerating tissues*

Some cells from the rings started to grow and attach to the surface of the plates. These cells were maintained in low glucose Dulbecco's modified Eagle's medium (DMEM; Gibco, Scotland) supplemented with 15% fetal bovine serum (FBS; Gibco, Scotland) and 1% penicillin and streptomycin (Biosera, UK), and incubated at 37°C in a humidified atmosphere containing 5% $CO_2$. About one week later, adherent cells which were confluent, were detached using 0.25% trypsin-EDTA (Gibco, Scotland) and transferred into 25-$cm^2$ culture flasks. The culture medium was refreshed every 2 days and cells were passaged twice a week. These cells were designated as SLCs. The cells were frozen in liquid nitrogen using freezing solution, containing 10% dimethyl sulfoxide (DMSO, Merck, Germany) and 90% FBS (Gibco, Scotland).

### *Karyotyping analysis*

In order to karyotype the cells, they were first

treated with vinblastine solution (0.5 µg/ml) (Sobhan Oncology Company, Iran) for 4 h and then detached from the flasks using trypsin-EDTA. Then KCl (0.56 g /100 ml $ddH_2O$) (Merck, Germany) was added to the cells and cold acetic acid/methanol solution (Merck, Germany) (3:1) was used to fix them. The cells were finally stained with 20% Giemsa solution (Merck, Germany) and analysed by light microscopy (Olympus, Japan).

***RNA extraction and RT-PCR analysis***

Total RNA was extracted from the SLCs at passage 5 using RNX-Plus solution (CinnaGen, Iran), according to the manufacturer's guidelines. The RNAs were then treated with DNaseI (5Prime, Germany), and their concentrations were determined using a NanoDrop spectrophotometer (Thermo Scientific, USA). 1 µg of the total RNA was used for reverse transcription in a 20 µl mixture containing 1 µl of Oligo(dT) primer (10 pmol) (Promega, USA), 2 µl dNTPs mix (10 mM) (CinnaGen, Iran), 4 µl cDNA synthesis buffer, 1 µl RNase inhibitor (40 u/µl) (Fermentas, Germany) and 1 µl M-MuLV reverse transcriptase (200 u/µl) (Fermentas, Germany) and DEPC-treated water (CinnaGen, Iran) to 20 µl. The reverse transcription was conducted at 42˚C for 1 h followed by 10 min incubation at 72˚C and samples were stored at -20˚C until use. The test cDNAs were normalised with controls using β-actin primers (Matin et al., 2004), and equal amount of each cDNA was used as template for PCR amplification with specific primer pairs, derived from the conserved regions of the reported sequences of human, rabbit and rat genes (*Oct4* and *Sox2*). The forward and reverse primer sequences are shown in table 1. Finally, 8 µl of PCR products were separated on a 1.2% agarose gel, stained with ethidium bromide, and visualised under UV light. The expression levels were compared to mRNA levels of desired genes in NTERA2 cells (a generous gift from Professor Andrews, University of Sheffield) and human adipose tissue derived mesenchymal stem cells (AT-MSCs), which were derived in the lab as described elsewhere (Ahmadian Kia et al., 2011).

***Flow cytometry analysis***

For flow cytometry analysis, cells were detached after treatment with trypsin-EDTA for 5 min at 37°C, washed with phosphate buffered saline (PBS) containing 5% FBS for three times, and fixed with 4% paraformaldehyde/PBS (Sigma, Germany) for 20 min at 4°C. After three times washing, the cells were permeablised with 0.2% digitonin (Sigma, Germany) for 10 min at 4°C and stained with Oct4 primary antibody (1:50) (Santa Cruz, USA) for 45 min at 4°C. The cells were then washed three times and incubated with FITC-conjugated anti-mouse IgG for another 30 min at 4°C. After final wash, cells were subjected to flow cytometry analysis using FACS Calibur (BD, USA) machine. Also, cells incubated with FITC-conjugated secondary antibody, in the absence of primary antibody were used as negative control. HFF3 cells (a generous gift from Royan Institute, Tehran, Iran) were also used as a control in this experiment.

## Results

***Derivation and culture of stem like cells (SLCs) from rabbit pinna***

The punched O-shaped rings from male New Zealand rabbit pinnas (figure 1) were able to produce outgrowth cells after 7-10 days in culture. The cells had a spindle like morphology, similar to Mesenchymal Stem Cells (MSCs) (figure 2). The growth curve of cells at passage 5 was analysed for 6 days in the media containing different percentages of FBS (figure 3). SLCs could grow better in DMEM medium supplemented with 15% FBS. The cells were grown for over a year (more than 120 passages) in the lab without obvious changes in their growth rate and morphology. They were also subjected to karyotyping analysis at passage 65, where no detectable abnormality was observed (figure 4).

***Stemness factors are expressed in SLCs derived from rabbit pinna***

The very well known stem cell molecular markers of Oct4 and Sox2 were shown to be expressed in these cells at mRNA level by RT-PCR. Meanwhile, the overall level of expression for *Oct4* was lower than that in the pluripotent NTERA2 cells and multipotent human adipose derived mesenchymal stem cells (figure 5). Although the level of *Sox2* mRNA expression was lower than that in the NTERA2 cells, but it seemed to be higher than its expression in AT-MSCs (figure 5). Expression of *Oct4* was further verified at protein level. Flow cytometry analysis revealed that 20% of the cells were Oct4 positive (figure 6A) which was far more than the human fibroblast cells (HFF3) which were used as control (figure 6B).

**Table 1.** Primer sequences used in RT-PCR experiments.

| Gene Name | Forward primer | Reverse primer | Amplicon (bps) | No. of Cycles | Annealing temp. (˚C) |
|---|---|---|---|---|---|
| *β-actin* | 5'- ATCTGGCACCACACCTTCTA CAATGAGCTGCG- 3' | 5'-CGTCATACTCCTGCTTGCTG ATCCACATCTGC-3' | 838 | 28 | 62 |
| *Oct4* | 5'- GAACATGTGTAAGCTGCGGCC-3' | 5'- CCCTTCTGGCGCCGGTTAC -3' | 270 | 40 | 58 |
| *Sox2* | 5'- AGCATGATGCAGGACCAG-3' | 5'- GGAGTGGGAGGAAGAGGT-3' | 269 | 40 | 52 |

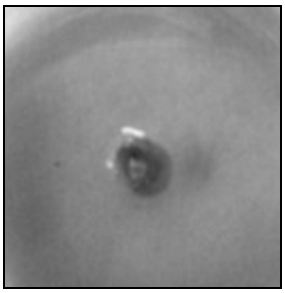

**Figure 1.** The O-shaped rings as punched from rabbit pinna in culture medium.

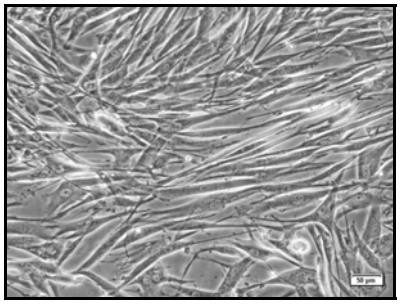

**Figure 2.** Morphology of SLCs in fifth passage (scale bar: 50 µm).

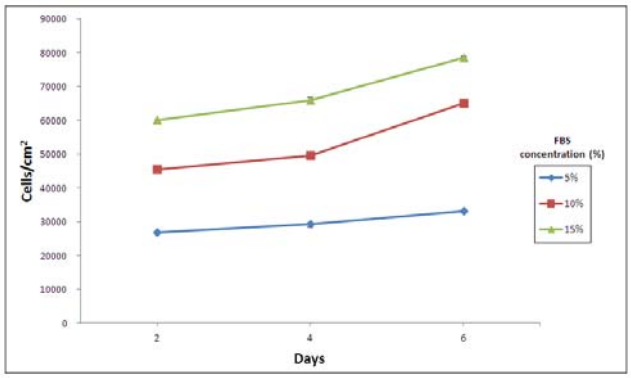


**Figure 3.** Growth rate analysis of SLCs grown in media containing various percentages of FBS, as examined by counting the cells at different time points (data are shown as Mean+/-SD).

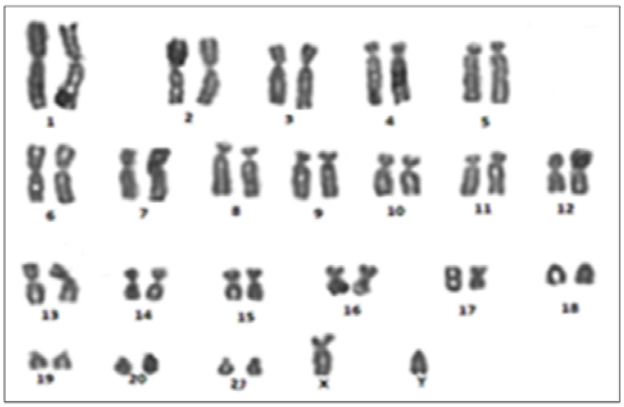

**Figure 4.** Karyotype analysis of SLCs, derived from male New Zealand white rabbit pinna at passages 65.

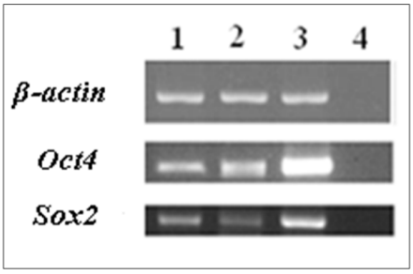


**Figure 5.** Semi-quantitative RT-PCR analysis of mRNA level for *β-actin*, *Oct4*, and *Sox2* genes. 1: SLCs 2: rat MSCs, 3: NTERA-2 cells, 4: non-template negative control.

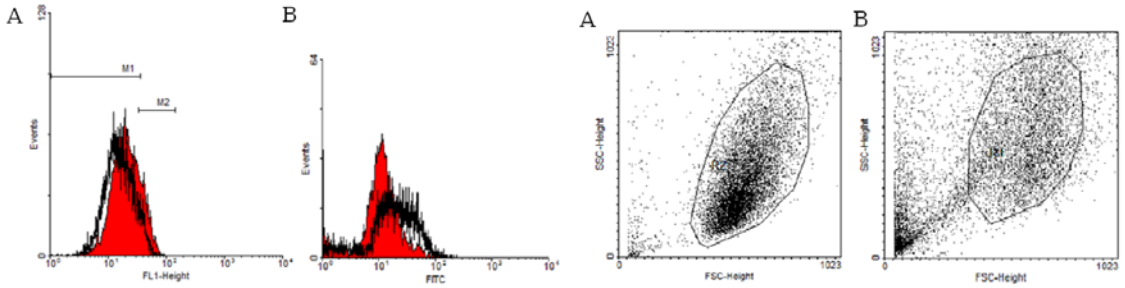


**Figure 6.** Dot plots (upper panel) and histograms (lower panel) of flow cytometry analysis for Oct4 protein expression in SLCs (A), and human fibroblast cells (HFF3) (B).

## Discussion

In biology, regeneration is a process in which a lost or damaged part of the body could heal so that the original function is restored. Some vertebrates like newts can regenerate injured limbs in two major steps, first dedifferentiation of the adult cells to a state similar to embryonic cells and second, development of fresh tissues from these cells (Odelberg, 2004). Simpler animals, like planarian have been shown to be far more efficient in regeneration by retaining clusters of stem cells within their bodies that migrate to the damaged parts and differentiate to provide the required missing tissues (Agata and Umesono, 2008). Beside these famous examples, tissue regeneration, although in lower extent, has also been reported in vertebrates including, salamander, zebrafish and few mammals like deer antler, mouse fingertip, MRL mouse and rabbits. A well known model for epimorphic regeneration in mammals is rabbit ear tissue regeneration, where all of the removed tissues are healed without any scar. This phenomenon was at first discovered by Markelova in 1953 and has been reviewed regularly since then (Joseph and Dyson, 1966; Goss and Grimes, 1972; Williams-Boyce and Daniel, 1980). This scarless regeneration was attributed to unique features of blastema tissue, with less-differentiated local cells, which proliferate and differentiate to new sheet of cartilage as it regenerates in a centripetal direction (Goss and Grimes, 1975). Since cells inside this tissue can establish all of the lost cells, we could use them in the field of regenerative medicine and also blastema tissue can be considered as a pool of powerful unique cells.

Having this interesting background, current study was aimed to culture these cells *in vitro* and determine their possible long term immortality in one hand and characterise their stemness state using the defined criteria based on stem cell markers expression on the other hand. These cells were successfully derived from the 2-day old ear rings. The number of passaging of these cells was phenomenal, reaching more than 120 passages until this report, without obvious changes in their morphology and growth rate. They kept these features even in the samples grown from the frozen cells. Expression of genes such as *Oct4* and *Sox2*, which are characteristic for ESCs, was detected in the derived SLCs.

In summary, to our best of knowledge this is the first time that immortal cells, sharing characteristics of pluripotent stem cells are derived from rabbit ears. These cells, which are designated as stem like cells (SLCs) in here, would serve as a good model for developmental biology studies as well as human disease preclinical tests. However, their characteristics need to be explored in more details.

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