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STUDYING THE EXPRESSION PATTERNS OF OCT4 AND SOX2 PROTEINS IN REGENERATING RABBIT EAR TISSUE

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Abstract: Epimorphic regeneration in New Zealand rabbit ear is an interesting example of mammalian wound healing in which blastema formation is involved in replacement of injured tissues. It has been suggested that isolated cells from regenerating rabbit ear possess stem-like properties. In this study, we aimed to determine the expression of stemness markers, OCT4 and SOX2 proteins, in regenerating rabbit tissues by immunohistochemistry. Results indicated that both proteins could be detected in epithelial cells, hair follicle cells and perichondrium cells. Expression pattern analysis of OCT4 and SOX2 proteins showed no clear differences between regenerative and non-regenerative control tissues. According to several reports of OCT4 and SOX2 proteins expression in adult stem cells, it could be proposed that OCT4 and SOX2 expressing cells in regenerating rabbit ear tissues are progenitor/adult stem cells which are resident in these tissues, and other markers should be used for detection of blastema cells.

Key Words: regenerating rabbit ear, OCT4, SOX2, adult stem cells.

INTRODUCTION

In higher organisms, response to traumatic injuries is achieved by either wound repair or wound regeneration (Clark *et al.,* 1998). In humans, skin trauma activates the repair process. Released cytokines and growth factors from inflammatory cells determine the function of cells present in the wound area (O'Kane and Ferguson, 1997; Ferguson and O'Kane, 2004). Fibroblasts are responsible for production of some extracellular matrix (ECM) proteins, tissue remodelling and scar formation (Singer and Clark, 1999). On the other hand, few vertebrate species are able to regenerate some of their damaged organs both structurally and functionally. Some examples include rabbit ear regeneration (Goss and Grimes, 1975), limb regeneration in xenopus (Goss and Holt, 1992), ear regeneration in MRL mouse (Clark *et al.,* 1998), deer antler regeneration (Allen *et al.,* 2002) and regeneration of deep soft tissues in bottlenose dolphin (Zasloff, 2011).

Epimorphic regeneration has been divided into 2 classes. In the first class, formation of blastema tissue –consisting of multipotent cells– leads to wound healing. Lost tissues are replaced by proliferation, migration and differentiation of blastema cells (Morgan, 1901). The second class applies other mechanisms including transdifferentiation, dedifferentiation and/or stem and progenitor cells for regeneration process; it does not involve blastema formation (Sanchez Alvarado, 2000).

Various publications suggest that blastema contains dedifferentiated (Bellairs and Bryant, 1985; Carlson, 2011) or likely undifferentiated cells (Butler, 1935; Gurley and Alvarado, 2008; Tweedell, 2010), whereas different appendage

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regeneration studies showed that blastema tissues consisted of a heterogeneous population of different lineage restricted stem/progenitor cells (Kragl *et al.,* 2009; Rinkevich *et al.,* 2011; Lee-Liu *et al.,* 2014). The later cell types have various origins and are not capable of differentiating into cells originated from other germ layers (Kragl *et al.,* 2009; Rinkevich *et al.,* 2011).

One of the first regeneration examples discovered in mammals was full regeneration of holes made in rabbit ear (Vorontsova and Liosner, 1960). Histological studies on healing process of rabbit ear were performed by Goss and Grimes (1975). They believed that chondroepithelial interactions resulted in epidermal downgrowth and this event avoided scar formation (Goss and Grimes, 1975; Goss, 1987). A similar model of regeneration was described for punched ear holes of MRL-MpJ mouse strain (Clark *et al.,* 1998). Ear scar-free regeneration accompanies blastema formation and tissue remodelling, including regrowth of cartilage and reformation of hair follicles and sebaceous glands (Clark *et al.,* 1998; Rajnoch *et al.,* 2003; Metcalfe and Ferguson, 2005).

A tissue or organ must maintain a balance between cell birth and cell loss, which is achieved by cells capable of self-renewal, differentiation and apoptosis. These cells, progenitor and stem cells, are the heart of the regeneration process (Metcalfe and Ferguson, 2008). Tissue specific stem cells were first described by Lajtha *et al.* (1964). They introduced these cells as multipotent stem cells that are capable of self-renewal and differentiation to more progenies (Lajtha *et al.,* 1964).

OCT4 and SOX2 are pluripotent stem cell markers. OCT4 belongs to the POU domain family of transcription factors. The members of this family bind to the octamer motif, ATGCAAAT, of target genes (Herr and Cleary, 1995). SOX2 transcription factor is a member of SRY-related HMG box family. OCT4, SOX2 and NANOG are the core network of transcription factors that maintain pluripotency state of embryonic stem cells (Nichols *et al.,* 1998; Avilion *et al.,* 2003; Chambers *et al.,* 2003). Ectopic expression of OCT4, SOX2, KLF4 and c-MYC can derive somatic cells towards a pluripotent state (Takahashi and Yamanaka, 2006). Soufi *et al.* (2012) suggested that OCT4, SOX2 and KLF4 could mediate chromatin remodelling, allowing the expression of specific genes which were critical for establishment and maintenance of the induced-pluripotent stem (iPS) cells pluripotency network (Soufi *et al.,* 2012). c-MYC increases the transcription level of all active genes and functions as a universal amplifier. It might help to maintain the activated state of the genes that are transiently expressed by reprogramming factors during the iPS cells generation (Nie *et al.,* 2012).

Mahmoudi *et al.* (2011) showed that cells derived from regenerating rabbit ear had stem-like properties and expressed *OCT4* and *SOX2* at mRNA level *in vitro* (Mahmoudi *et al.,* 2012). In this study, we aimed to trace these cells in the regenerating rabbit ear tissue by analysing the expression patterns of OCT4 and SOX2 proteins.

MATERIALS AND METHODS

Animals

A group of 3 male New Zealand white rabbits (*Oryctolagus cuniculus*) aged between 2 and 3 mo and a group of 3 male Wistar rats (*Rattus norvegicus*), 8-10 wk of age, were used as regenerative and non-regenerative mammalian models, respectively.

Animals were purchased from Razi Vaccine and Serum Research Institute (Mashhad, Iran), and kept under conditions of 12 h light and 12 h darkness and standard nutrition for at least 1 wk before sampling. All experiments were performed according to protocols established by Animal Research Committee of Ferdowsi University of Mashhad.

Preparation of tissue samples

After shaving the ears, 10% lidocaine (Iran Daru) was used for local anaesthesia in order to painlessly extract punches from rabbit pinnas. Both ear surfaces and a metal puncher were cleaned with 70% ethanol (Merck). Primary through-and-through holes were made with 2 mm diameter. Punches were taken near the central area of the ear, while avoiding major blood vessels. Four millimetres O-shaped rings of ear tissue were obtained by secondary punches at 0, 2 and 12 d after the primary cuts (Goss and Grimes, 1975; Mahmoudi *et al.,* 2012).

Anesthetising the rats was done by injection of xylazine (Alfasan) at 10 mg/kg in combination with ketamine (Alfasan) at 50 mg/kg and samples were prepared 2 d after the first punches.

Tissue samples were fixed in 10% formalin (Sigma) for 16-20 h, dehydrated in increasing concentrations of ethanol, embedded in paraffin (Histo Line Laboratories), cut into sections with 6 µm thickness and finally transferred onto polylysinated slides.

Immunostaining

Antibodies

The following antibodies were used for immunohistochemical detection of OCT4 and SOX2 in this study. For OCT4, mouse monoclonal antibody (C10, Santa Cruz, sc-5279) against amino acids 1-134 of human OCT4 protein and HRP-conjugated polyclonal antibody against heavy chain of mouse IgG2b (Goat IgG2b-HRP, Abcam, ab-97250) were used as primary and secondary antibodies, respectively. To analyse SOX2 expression, recombinant monoclonal antibody (10F10, Novus Biologicals, NBP1-51688) against human SOX2 protein and HRP-conjugated polyclonal antibody against heavy chain of IgG1 (Goat IgG1-HRP, Abcam, ab-97240) were used as primary and secondary antibodies, respectively.

As no specific antibodies are commercially available for rabbit's OCT4 and SOX2 proteins, anti-human antibodies were applied in this study. Alignment of human and rabbit OCT4 proteins revealed 87.22% identity between the 2 sequences. However, less identity (80.60%) was observed when the first 134 amino acids –against which the primary antibody was raised– of the 2 proteins were compared. So, we predicted that this antibody could probably detect rabbit OCT4 protein. In the case of SOX2 antibody, because of 97.18% identity between the amino acid sequences of human and rabbit SOX2 proteins, there is a high probability that the primary antibody against human SOX2 would also be able to identify rabbit SOX2 protein (Figure 1).

Immunohistochemistry

Sections were stained by immunohistochemical method. Samples were rehydrated in attenuating concentrations of ethanol. 10 mM sodium citrate (pH: 6) was used as antigen retrieval solution for 30 min at 100°C. After cooling to 50°C, sections were washed 3 times in tris-buffered saline (TBS) for 5 min. The specimens were then treated with 3% H_2O_2 (Sigma) for inactivation of endogenous peroxidase at room temperature for 15 min. Then, the samples were blocked in 4% bovine serum albumin (BSA, Invitrogen) in TBS solution for 45 min at room temperature. Sections were kept in a humidified chamber and incubated with primary antibody (diluted 1:100 for anti-OCT4 and 1:200 for anti-SOX2) for 16 h at 4°C. Negative controls were run without primary antibodies. Samples were rinsed in TBS 3 times and incubated with HRP- conjugated secondary antibody (diluted 1:800) at room temperature for 1 h. Finally, the samples were washed in TBS, and 3,3'-diaminobenzidine (DAB, Sigma) was applied for visualisation and Harris haematoxylin (Novocastra) was used for counterstaining. The sections were dehydrated and mounted with glue (Entellan, Merck). The photographs were taken using Olympus DP71 camera coupled to an Olympus EX51 microscope.

RESULTS AND DISCUSSION

Prepared sections from rabbit pinnas (regenerative tissue) on days 0, 2 and 12 and from rat ear (non-regenerative tissue) on day 2 after punching were examined for OCT4 and SOX2 expression by immunohistochemical staining. New Zealand rabbit ear is a well-known example of mammalian regeneration (Vorontsova and Liosner, 1960) whereas Wistar rats are not able to close their punched ears.

At day 0, samples of rabbit ear showed that epithelial, hair follicle and perichondrial cells expressed both OCT4 and SOX2 proteins (Figure 2).

Scab –including blood clots and necrotic tissues– was seen in sections prepared 2 d after the first punch in rabbit pinnas, and epithelial cells and hair follicle cells expressed both proteins. The patterns of OCT4 and SOX2 expression were identical with 2-d rat ear tissue samples (Figure 3).

Figure 1: The amino acid sequences of OCT4 and SOX2 proteins. Human OCT4A sequence (Uniprot accession number, Q01860) is aligned with the sequence of rabbit OCT4 (Uniprot accession number, A0A0B4UDY6) (A). Alignment of SOX2 amino acid sequences of human (Uniprot accession number, P48431) and rabbit (Uniprot accession number, A0A0B4UET6) (B). OCT4A, Octamer-binding transcription factor 4 isoform A; SOX2, SRY (sex determining region Y)-box2.

Punched holes were almost closed in 12-d samples of rabbit ear. Re-epithelialisation and downgrowth of epithelial cells could be observed clearly. There was an irregular border between the epidermis and its lower dermis region. Proliferating epidermal and perichondrial cells expressed OCT4 and SOX2 markers. In the region far from closing hole in these samples, some of epidermal cells and follicle cells of dermis also expressed OCT4 and SOX2 proteins (Figure 4, C-D, arrows demonstrate these regions).

Our results indicated no differences between expression patterns of OCT4 and SOX2 proteins in regenerative rabbit ear and non-regenerative rat ear on 2-d tissue samples.

Epidermal and perichondrial cells were proliferating on day 12 after punching rabbit ear tissues, and the boundary between epidermal cells and adjacent dermis was very irregular. Such structures have been observed in healing ears of MRL mouse, while punched holes in the pinna of C57BL/6 mouse did not show this event (Gourevitch *et al.,* 2003).

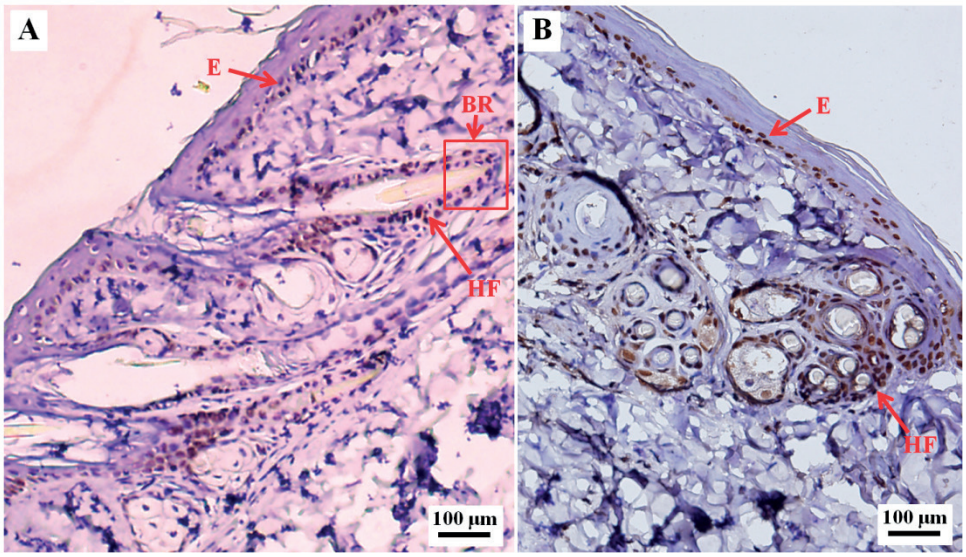

Figure 2: Immunostaining of regenerating rabbit ear tissues at day 0. Epidermal and hair follicle cells expressed OCT4 (A) and SOX2 (B) proteins. E, epidermal cells; HF, hair follicle cells; BR, bulge region. Scale bars, 100 µm.

Heber-Katz described differences between basement membrane and extra cellular matrix (ECM) in healing wounds of MRL and C57BL/6 mice. She believed that basement membrane and ECM degradation helped blastema formation in healing ear of MRL mouse, whereas basement membrane remained unchanged in injured C57BL/6 mouse and a scar was formed (Heber-Katz, 1999).

It is notable that proliferating perichondrial cells (Figure 4, G-H) express both OCT4 and SOX2 proteins. This suggests that perichondrial cells contribute to blastema formation in regenerating rabbit ear. These cells were considered precursors of blastema formation because of their capacity for proliferation, migration and differentiation (Williams-Boyce and Daniel, 1986).

Adult stem cells are responsible for homeostasis of tissues in which they are resident. They are rarely divided in normal states (Alonso and Fuchs, 2003). As these cells are responsible for all cell replacements in a tissue, they necessarily contribute to wound healing. These cells are placed in special niches in a tissue and are exposed to several differentiation signals (Metcalfe and Ferguson, 2008).

Self-renewal and multipotency are the 2 characteristics of adult stem cells (Lajtha *et al.,* 1964). Keratinocytes have such potential within the skin tissue. They could be maintained and proliferated *in vitro* (Rheinwald and Green, 1975; Rheinwald and Green, 1977). Hair follicle bulge is another niche of the skin somatic stem cells (Stenn and Cotsarelis, 2005). These cells play a regenerative role for injured skin, as they contribute to producing epidermis, hair follicle and sweat glands in the injured area (Zheng *et al.,* 2005).

LRCs (Long term label- retaining cells) were identified in the perichondrium part of adult rabbit ear cartilage. These cells had a longer cell cycle than transient proliferating cells (Togo *et al.,* 2006). LRCs were also found in ear perichondrium of adult mouse and were introduced as progenitor/ adult stem cells (Kobayashi *et al.,* 2011).

Whereas OCT4 and SOX2 are known as pluripotency markers, their expressions have also been examined in somatic stem cells. Tai *et al.* showed that adult stem cells expressed OCT4 in several human tissues such as liver, kidney and pancreas (Tai *et al.,* 2005). OCT4 expression was also reported in side populations of keratinocytes in mouse epidermis (Redvers *et al.,* 2006) and resident cells in the human hair follicle bulge (Yu *et al.,* 2006). Some studies

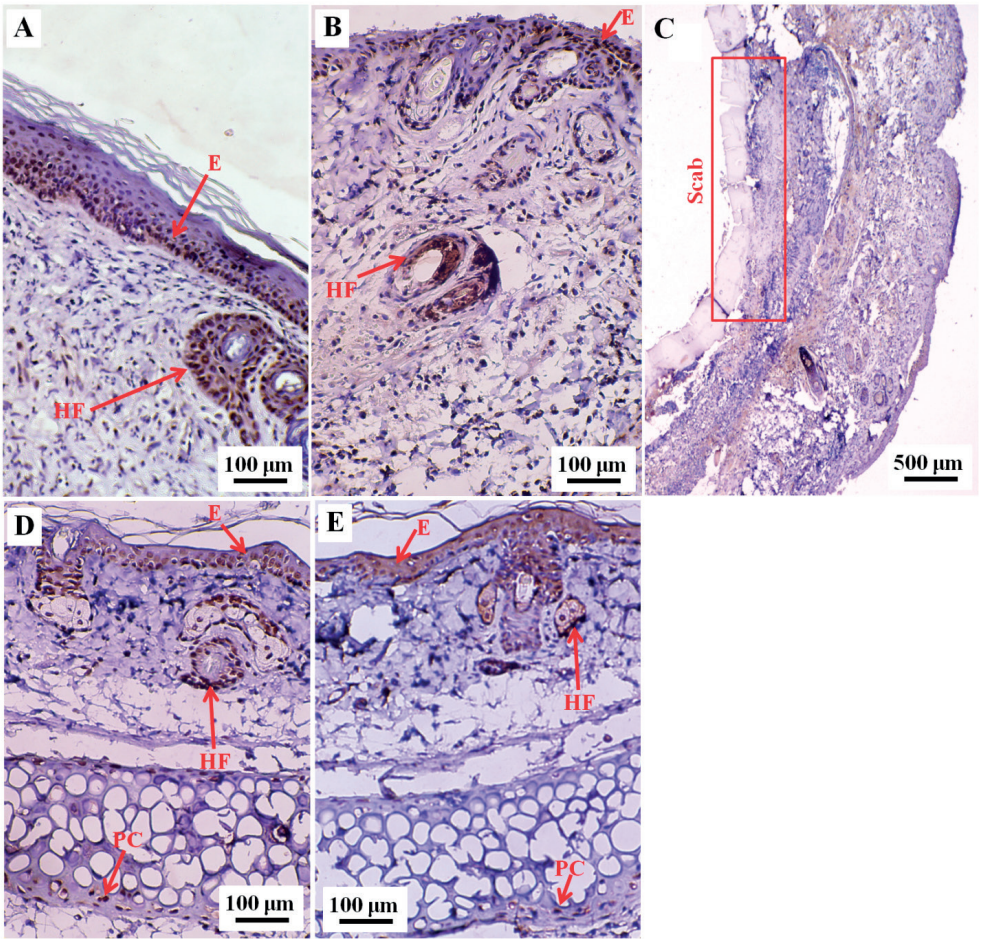

Figure 3: Immunostaining of regenerating rabbit ear tissues and non-regenerative rat ear tissues at day 2. Epidermal, hair follicle and perichondrial cells expressed OCT4 (A, D) and SOX2 proteins (B, E). The scab area including blood clot and necrotic tissues is indicated (C). A, B and C demonstrate sections related to rabbit ear while D and E represent rat ear sections. E, epidermal cells; HF, hair follicle cells; PC, perichondrial cells. Scale bars, 100 µm (A, B, D and E) and 500 µm (C).

addressed OCT4 protein expression in mesenchymal stem cells from different origins (Izadpanah *et al.,* 2006; Greco *et al.,* 2007; Riekstina *et al.,* 2009; Dudakovic *et al.,* 2014).

Sarkar and Hochedlinger (2013) found that tissues which require SOX2 for foetal development maintain expression of this transcription factor in some progenitor/adult stem cells derived from those tissues (Sarkar and Hochedlinger, 2013). SOX2 expressing progenitor cells have been identified in retina (Taranova *et al.,* 2006), trachea (Que *et al.,* 2009), tongue epithelium (Okubo *et al.,* 2009) and hair follicle (Driskell *et al.,* 2009). Isolated human dermal progenitor/stem cells demonstrated high proliferation capacity and high level of SOX2 expression. These cells could differentiate not only to mesodermal cells such as adipocytes, chondrocytes and osteoblasts, but also to neural cells (Shim *et al.,* 2013). Immunohistochemical studies of peripheral nervous system showed that immature and dedifferentiated Schwann cells expressed SOX2 protein in adult rats (Kioke *et al.,* 2014). Táncos *et al.* (2015)

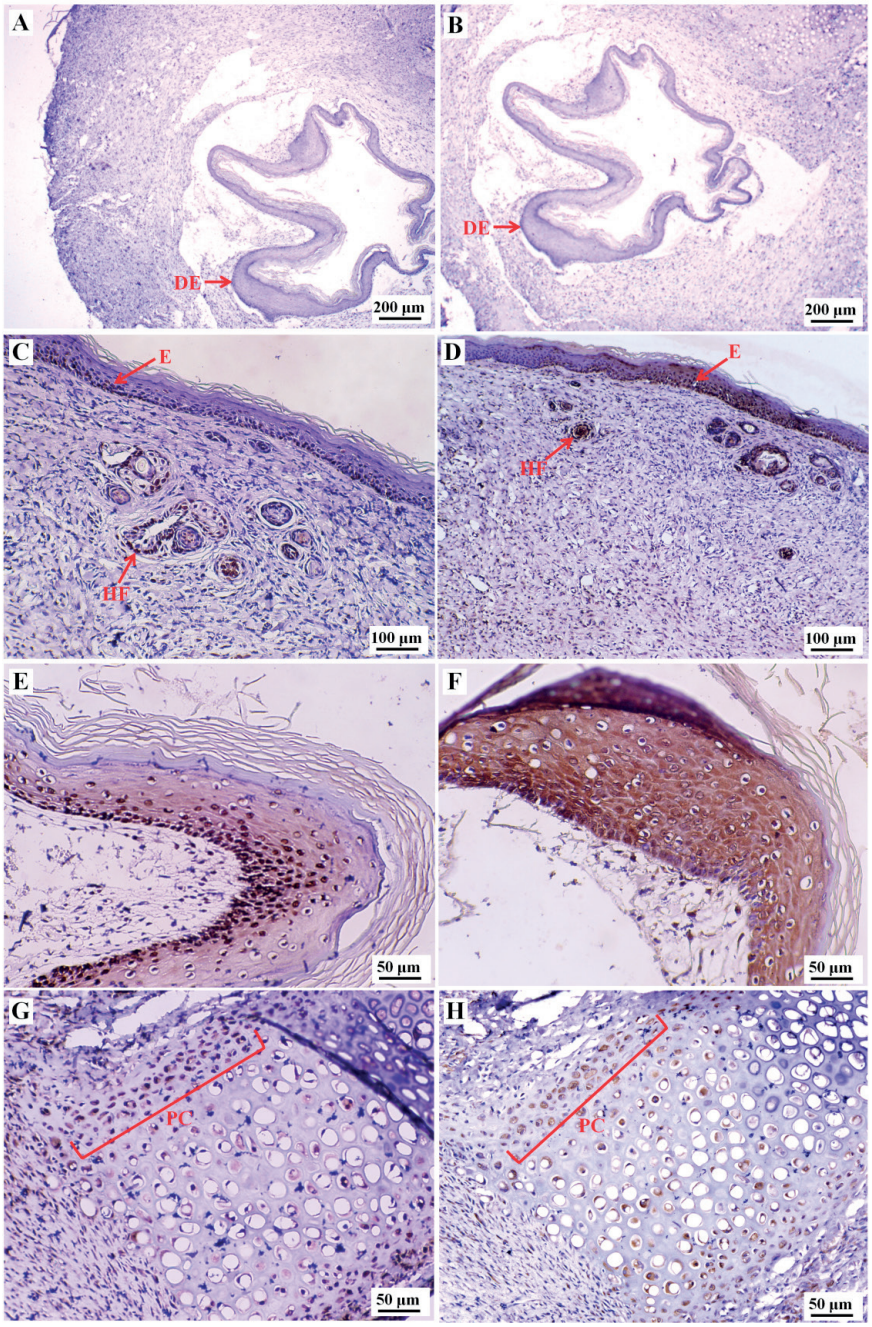

Figure 4: Immunostaining of regenerating rabbit ear tissues at day 12. Downgrowth epithelialisation and negative controls for OCT4 (A) and SOX2 (B) proteins. Epidermal and hair follicle cells expressed OCT4 (C) and SOX2 (D) proteins. Cells in re-epithelialisation region expressed both OCT4 (E) and SOX2 (F) proteins. Proliferating perichondrial cells expressed OCT4 (G) and SOX2 (H) proteins. DE, downgrowth epithelialisation; E, epidermal cells; HF, hair follicle cells; PC, perichondrial cells. Scale bars, 200 µm (A, B), 100 µm (C, D) and 50 µm (E, F, G and H).

suggested that OCT4 and SOX2 proteins might play conserving roles in maintaining and renewing various organs in the rabbit (Táncos *et al.,* 2015).

It is also likely that anti-OCT4 antibody which is used in this study could not function specifically and has produced false positive signals related to other antigens. A previous study used an antibody (C10, Santa Cruz, sc-9081) for detection of amino acids 1-134 of OCT4 stemness marker in the testis-derived cells of marmoset monkey. Similar to our results, this antibody showed nuclear and also weak cytoplasmic signals (Warthemann *et al.,* 2012). The authors suggested that this antibody was not capable of distinction between OCT4A and OCT4B isoforms and could also detect an unrelated epitope to OCT4 (Bhartiya *et al.,* 2010; Warthemann *et al.,* 2012). OCT4A is a nuclear protein that is involved in stemness and pluripotency, while OCT4B isoform is mainly present in cytoplasm and it is related to stress response of the cells (Cauffman *et al.,* 2005). More recently, Táncos *et al.* (2015) also applied monoclonal antibody (C10, Santa Cruz, sc-5279) to identify OCT4 protein in rabbit embryonic stem cells, although they were not working with tissues or a mixed population of cells (Táncos *et al.,* 2015).

CONCLUSIONS

In conclusion, it could be suggested that OCT4 and SOX2 expressing cells in regenerating rabbit ear tissues are progenitor/adult stem cells which exist in these tissues. We propose that the expression of OCT4 and SOX2 should be verified and compared between regenerative and non-regenerative tissues at other time points between 2 and 12 d, or that other markers are required to specify blastema cells in regenerating rabbit tissues.

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