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RESEARCH OPINIONS IN ANIMAL & VETERINARY SCIENCES

Isolation, culture and characterization of foetal tendon stem cells (FTSCs) in rabbit

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Abstract

This study aimed for isolation, culturing and characterization properties of foetal tendon stem cells (FTSCs) derived from rabbit foetal tendons. TSCs were isolated from patellar and Achilles tendons of foetal rabbits. These TSCs were examined for their stemness property. The morphological characteristics of the TSCs were chronologically studied. The cell marker expression of the cells was examined using histochemical and immunohistochemical analysis. In addition, morphology, colony formation and proliferation of TSCs were also compared. Histochemical analysis of cells was done with a polychromatic stain Giemsa which revealed the morphological characteristic of the fixed cells. Immunohistochemical analysis of TSCs was done for the detection of the stem cell markers octamer-binding transcription factor 4 (Oct-4) and stage-specific embryonic antigen-4 SSEA-4. The TSCs cultured from foetal tendons via patellar tendon and Achilles tendon expressed the stem cell markers Oct-4 and SSEA-4 and also exhibited the typical morphology of tendon stem cells. Both small and large colonies were seen on the culture plate and stained with Giemsa. Morphologically, TSCs possessed smaller cell bodies and larger nuclei. This study showed that TSCs exhibit distinct properties, including expression of cell markers (Oct-4 and SSEA-4), proliferative, differentiation potential and cell morphology in culture. The future application of these *in vitro*-cultured TSCs can be possibly done on the tendon tissue engineering for the repair of tendons using the cell application based therapy.

Keywords: TSCs; tissue engineering; foetal; tendon

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Introduction

There are about 30 million annual tendon and ligament injuries worldwide (Maffulli et al., 2003). Tendons were originally thought to consist of tenocytes only, however, a recent study has demonstrated that mammalian tendons also contain stem cells, referred to as tendon stem/cells (TSCs) (Zhang eta l., 2010). These cells are more suitable for the tendon tissue engineering. Injury of tendon is a very common problem with complicated healing outcomes. Foetal stem cells have great promise in enhancing the biologic healing, cross contamination and foetal cells have four basic properties i.e., i) intrinsic plasticity, ii) ability to grow and proliferation, iii) ability to produce growth factors and iv) reduced antigenicity. TSCs are characterized by their multi-differentiation potential, including differentiation into adipocytes, chondrocytes, and osteocytes (De Mos eta l., 2007). So these cells can have a great potential for tendon tissue engineering. Tendon tissues are also characterized by the presence of fibroblasts/fibrocytes (ligament) or tenoblasts/tenocytes (tendon) and an abundant extracellular matrix (ECM), mainly composed of collagen I (Gelse eta l., 2003), resulting in a dense and hypocellular structure. Therefore, tendon tissue engineering is receiving increasing attention as a potential strategy for the treatment of tendon injuries and can be attempted to improve the structure and function of injured (Butler et al., 2008).

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The tendon cell culture from the tendon includes tenocyte and tendon stem cells. Since TSCs have stemness properties (Zhang et al., 2011) to the common molecular processes underlying the core stem cell properties of self-renewal and the generation of differentiated progeny (Matthew, 2011). It is thus TSCs which would have a probable role in the regeneration, hence, this present study aims for isolation, culturing and characterization of foetal tendon stem cells (FTSCs) derived from rabbit foetal tendons Achilles and patellar tendon.

Materials and Methods

FTSCs were obtained from the foetuses of pregnant full term NZW rabbits after performing caesarean section.

Isolation and culture of FTSCs

Permission from the ethical committee was sought. One female rabbit with gravid uterus of 25 days old pregnancy was sedated using intra-muscular Xylazine (5 mg/kg body weight and Ketamine (50 mg/kg body weight) injection and then C-section was performed. (Fig. 1) After C-section, 4 foetuses were collected and sacrificed by using intra cerebral injection of xylazine.

Foetal patellar and Achilles tendons were dissected. Total 8 patellar and 8 Achilles tendons were collected from 4 foetuses. The middle portions of tendons were utilized for cell culture. The tendon sheath and surrounding paratenon were removed, and the middle tendon portion tissues were then washed three times in 1XPBS with 100U/ml penicillin and 100µg/ml streptomycin. After the antibiotics treatment, tissues were cut up into small pieces (1 mm × 1 mm × 1 mm), and washed with DMEM/F12 medium. Tissue sample was then digested with 1 mg/ml collagenase type I (MP biomedical) in 1 ml of DMEM/F12 (1:1) medium at 37°C for 18 hr or overnight. After digestion, DMEM/F12 with 10% FBS of the volume same as that of collagenase used was added and cells were then filtered with 70µm cell strainer. The suspension was centrifuged at 1,500 rpm for 15 min, the supernatant was discarded. Remaining cell pellet was re-suspended in growth medium consisting of Dulbecco's modified Eagle's medium (DMEM; Gibco-life technologies) supplemented with 20% foetal bovine serum (FBS; Gibco-life technologies), 100 U/ml penicillin and 100 µg/ml streptomycin (MP biochemicals) to obtain a single-cell suspension. Cell suspension was seeded in T25 flasks at 37°C with 5% CO_2. After 8-10 days in culture colonies were formed on the culture surface of the flask. The cell colonies were stained with methyl violet (Bi et al., 2007). Cells were passed once a week. In separate cultures, individual cell colonies were detached by treatment with prewarmed (37°C) Accutase, after leaving it for 5-10 min at 37°C 1-2 millilitre of DMEM/ F-12 with 10% FBS was added, the suspension was collected and subjected to centrifuge at 1500 rpm for 5 min. The pellet was then collected and dissolved in DMEM/F-12 medium

Gross morphology of FTSCs and their colonies

Gross morphology of FTSCs was confirmed by using inverted microscope (Olympus) for cultured cells in suitable medium as mentioned above after 2nd, 3rd and 4th days. Uniformly in all the flasks the morphology of the images remained constant. Methyl violet staining was used to study the morphology of TSC colonies as described (Bi et al., 2007).

Giemsa stain, which is a polychromatic stain consisting of a mixture of dyes of different hues which provide subtle differences in staining, was used to study the morphology of cells (Li et al., 2009). After removing culture medium, cells were washed with PBS 3 times, for 5 min each time. Cells were fixed in 70% ethanol (v/v) for 15 min and stained with a pre-mixed Giemsa staining solution for 30 min at room temperature. The stained cells were rinsed with tap water, air dried and examined under phase contrast microscope.

Immunohistochemical analysis of TSCs markers

Using immunocytochemistry, we examined the following stem cell markers: octamer-binding transcription factor 4 (Oct-4) and stage-specific embryonic antigen-4 (SSEA-4) as these are the indicators of stemness properties of any progenitor cell. TSCs were fixed with 4% paraformaldehyde in PBS for 20 min at RT and Permialization with 0.3% triton X used for 20 min at RT (for Oct-4), blocked with 5% BSA for 40 min at RT, reacted with mouse anti-human Oct-4 (1:200; Santacruz) and SSEA-4antibody (1:200; Santacruz) for overnight at 4°C. After washing the cells with PBS, FITC-conjugated goat anti-mouse IgG secondary antibody was applied for 40 min at room temperature for Oct-4 and SSEA-4 (1:500) and cells were also counterstained with 0.8 µg/ml of DAPI. Tenocytes were used as a control; the stained cells were examined by using fluorescence microscopy with appropriate filter combinations.

Results

Isolation and characterization of tendon stem cells

After 3-5 passages from primary culture it was possible to culture the TSCs using the standard protocol. Figure 2 represents the growth of primary culture and passages 1-3. In primary culture, no cellular adhesion was noted until 2nd day. After 3 day, growth resumed and cells grow in an exponential pattern. In primary culture, TSCs were elongated with central nucleus.

Morphology of FTSCs

It was noticed that TSCs after a dormant period of 3-4 days, started to form the first colony from the single cell

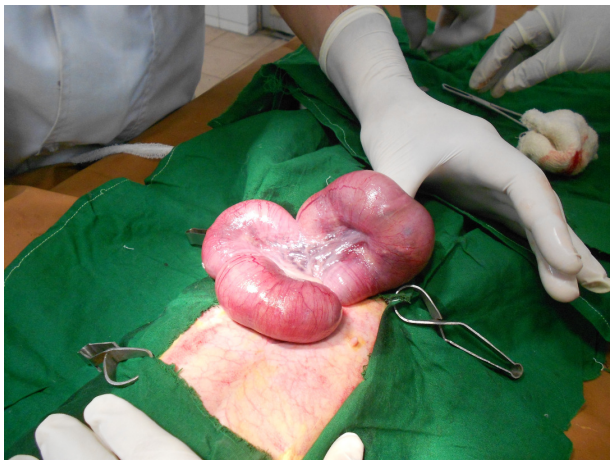

Fig. 1: Caesarean section in the female rabbit to obtain the foetuses

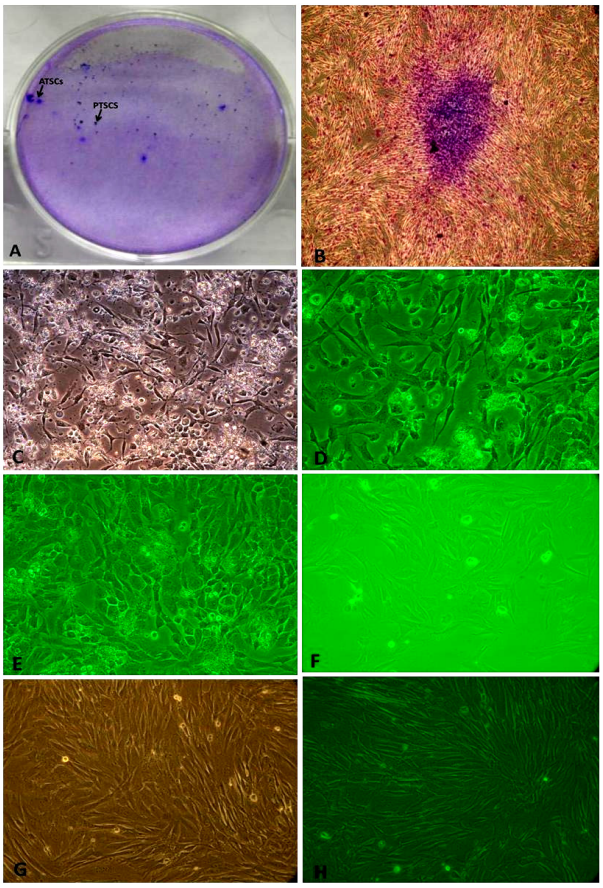

Fig. 2: The colony formation of rabbit tendon stem cells (TSCs), A. Total PTSC (Small) and ATSC (Large) colonies as arrows indicated, B. Morphology of a formed colony stained with Methyl violet at P1 second day, C, D and E are expended colonies of first, third and fourth days after seeding respectively for P 0. F. Showing TSCs P1, 10X, day 2 (colony), G. showing TSCs P1, 20X, day 3, H. TSCs P1, 20X, day 7 and D. showing TSCs P3,20X, day 2

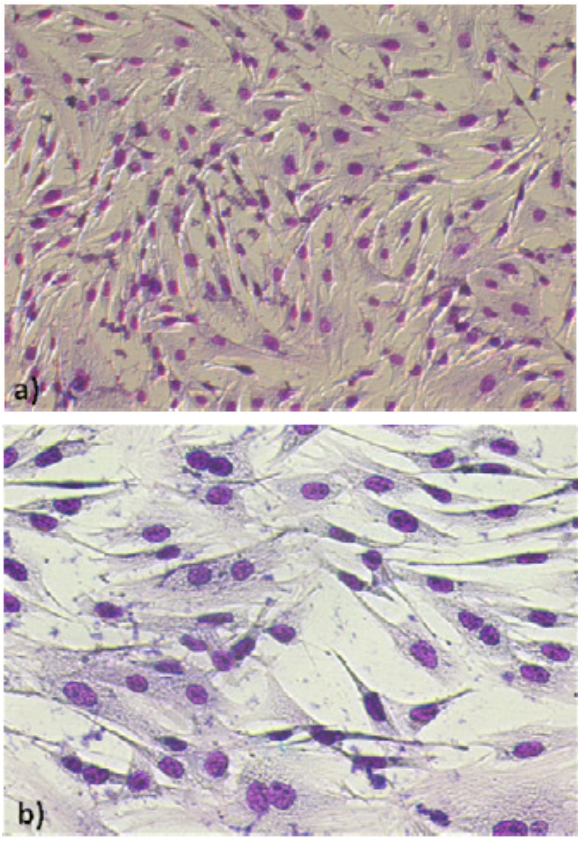

Fig. 3: Macrophotograph of Giemsa stained FTSCs culture, a) FTSCs at P1, 10x and b) FTSCs at P1, 20 x

at 3 days. By 10 days, numerous colonies were then formed and it was evident that TSCs formed more colonies (Small and Large). When the good number of colonies appeared in the culture flask (Fig. 2A), passing was preceded. The grown colonies exhibited different shapes and structures (Fig. 2A). At passage 1, the sizes of cells increased compared with that in primary culture. TSCs required 17 to 19 days to complete 3 passages (Fig. 2H). All TSCs positive colonies were visualized with methyl violet, (Fig. 2B). The colony-forming efficiency was evaluated by methyl violet staining. TSCs cell expansion yielded significantly. After purifying and seeding, Giemsa staining clearly revealed that the cells were mainly triangle or spindle shaped, with darker red cytoplasm, a round or elliptic pansy-purple nucleolus lying at centre or at the margins of the cell, and 1-2 clear nucleoli (Fig. 3).

Immunocytochemistry of TSCs

To find out the clonogenicity, multipotency and self-renewal of the FTSCs, Oct-4 and SSEA-4 presence wasexplored. These colonies were heterogeneous in size and cell density, potentially reflecting differences in the rate of cell proliferation (Fig. 2A and B). Immuno-

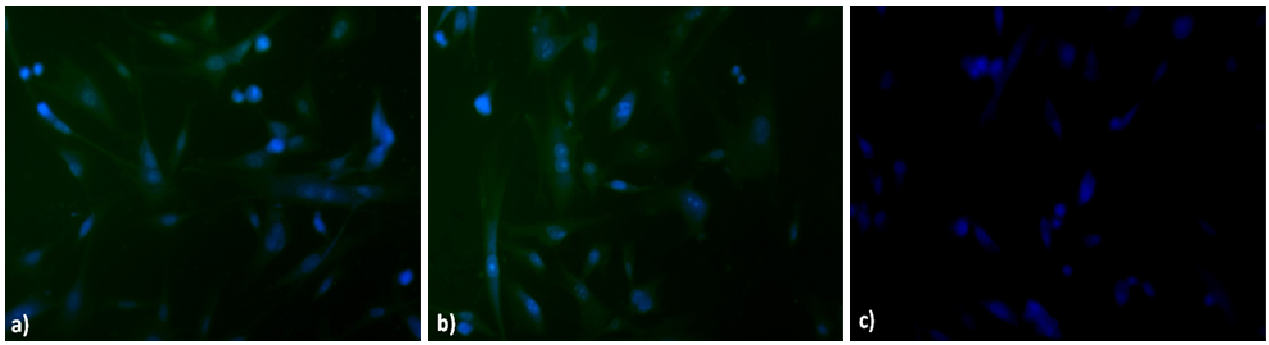

Fig. 4: The testing of Tendon stem cell marker expression. Immunocytochemical staining of TSCs for oct-4 and SSEA-4-4 fixed in PFA, a) Indicate TSCs at P3 expressed Oct-4, b) indicate TSCs at P3 expressed SSEA-4 and c) indicate TSCs at P3 (Positive control) no expression of Oct-4 and SSEA-4.

cytochemical staining of FTSCs showed that patellar and Achilles tendon in culture for more than 1-2 months at passages 3-5 expressed Oct-4 (Fig. 4a), SSEA-4 (Fig. 4b), and control (Fig. 4c). However, tenocytes exhibited an absence or very low levels of staining for these cell markers.

Discussion

This study described the culture of foetal rabbit TSCs in monolayer. The TSCs isolated were from the foetal Achilles tendon and patellar tendon. Latest view of the TSCs and its morphological observations was presented. Figure 2 represents the growth of primary culture from P1 to P3. In primary culture, no cellular adhesion was noted until 2nd day. This fact may explain the loss of cells. Clusters of cells expressing TSCs are also positive for methyl violet, a dye that labels cells containing high-level organelles. Immunofluorescent staining revealed that TSCs in culture have elongated profile with elongated nucleus. It also showed that FTSCs expressed Oct-4 and SSEA-4, which are known stem cell markers. The tenocytes were seen to be negative for these markers which indicate that tenocyte differs from tenoblast in stemness. This was in confirmation with the observations of Zhang et al. (2010). TSCs in culture, that the former exhibited a cobble-stone shape. Finally, TSCs proliferated significantly faster in culture. Oct-4 is a transcription factor that is typically expressed in embryonic stem cells during development and is essential for establishing and maintaining undifferentiated pluripotent stem cells (Donovan, 2001). Like previous studies that showed Oct-4 expression in human and mouse bone marrow stem cells (BMSCs)(Goolsby et al., 2003; D'Ippolito et al., 2004; Lamoury et al., 2006). It was also found that TSCs expressed Oct-4, encouraging future examination of whether the multi-potency of TSCs demonstrated in this study depends on Oct-4 expression. SSEA was consistently expressed in TSCs at low (<2) and high passages (~12) even after long term culturing. It is known that SSEA-4 is developmentally regulated during early embryogenesis and is widely used as a marker to monitor the differentiation of both mouse and human embryonic stem cells (Andrews et al., 1982; Henderson eta l., 2002). Therefore, SSEA-4may be used as one of TSCs markers. Thus, the high levels expression in rabbit TSCs indicate that TSCs were an actively proliferating, self-renewing population of cells in our culture conditions. Finally, as this study shows that TSCs, express Oct-4 and SSEA-4 they may be used as markers to detect these tendon stem cells in situ.

Conclusion

The results of this study shows that foetal tendon stem cells can be successfully cultivated from the patellar and Achilles tendons of rabbit foetus. They can be cultured and probably stored for future application which is yet to be investigated. The monolayer culture can also be employed for studying the functional physiology of tendon cells. The future application of these in vitro-cultured TSCs can be explored by in vivo application of these cells for tendon tissue engineering using the cell-scaffold interaction based therapy.

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