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Generation of handmade cloned embryos from adipose tissue derived mesenchymal stem cells in goat

Hruda Nanda Malik, Amit Kumar Dubey, Dinesh Kumar Singhal, Shravani Saugandhika, Sushil Kumar Mohapatra, Dhruba Malakar*

Animal Biotechnology Centre, National Dairy Research Institute, Karnal 132001, India

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ABSTRACT

The present study aims to isolate and culture adult mesenchymal stem cells (MSCs) from adult adipose tissues of goat followed by their thorough characterization and in vitro differentiation into osteocytes, chondrocytes and adipocytes. These cells were further used as donor nuclei for production of handmade cloned embryos. The goat adipose tissue-derived mesenchymal stem cells (ADSCs) were positive for the expression of CD29, CD34, CD44, CD90 and CD166 (MSCs specific positive markers) and negative for CD45 and CD71 (MSCs specific negative markers). The expression of specific markers for osteocytes (Osteopontin, Collagen I), chondrocytes (Aggrecan and Collagen II) and adipocytes (LPL, PPARα and PPARγ) was also detected from these cells. The present study reported no significant difference in cleavage, morulae and blastocyst production, when ADSCs (69.48 ± 0.86, 30.69 ± 1.09 and 10.96 ± 0.82), fetal (73.3 ± 1.26, 34.12 ± 0.79 and 13.86 ± 0.69) and adult (71.81 ± 0.64, 34.07 ± 1.00 and 11.71 ± 0.63) fibroblasts were used as somatic donor cells. On staining with Hoechst 33342 dye, a well-developed blastocyst revealed 90–100 numbers of blastomeres under an epifluorescence microscope. In the future, the ADSCs may be used as a convenient source for autologous therapy and production of transgenic animals.

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1. Introduction

Stem cell biology is one of the most discussed areas in biomedical research today. However, there is much controversy surrounding the use of embryonic stem cells in regenerative medicine due to their ethical constraints. This controversy has altered the focus of investigation to the isolation of alternative adult stem cell precursors. Diligent efforts of current investigations are now focusing on the isolation and characterization of somatic stem cells, popularly known as mesenchymal stem cells (MSCs). The MSCs are multipotent stromal cells capable of possessing two fundamental characteristics that are the ability of extensive replication and the capacity of multilineages differentiation into osteocytes, chondrocytes, adipocytes, neurocytes and myocytes. Mesenchymal stem cells have been isolated from bone marrow (Digirolamo et al., 1999), adipose tissue (Ren et al., 2012), synovial fluid (Suzuki et al., 2012), skeletal muscle (Jankowski et al., 2002), deciduous teeth (Kerkis and Caplan, 2012), lung (Noort et al., 2002) and umbilical cord (Gang et al., 2004). The MSCs were first identified in bone forming progenitor cells of rat bone marrow (Friedenstein et al., 1966). Bone marrow derived MSCs are multipotent, and thus suitable for tissue engineering and regenerative medicine. However, the derivation of bone marrow derived MSCs is a complex and painful procedure. On the other hand, adipose tissue derived MSCs are extremely abundant and easily accessible and yield high cell numbers to efficiently expand cell populations.

* Corresponding author. Tel.: +91 9416741839; fax: +91 184 2250042.
E-mail addresses: dhrubamalakar@gmail.com, dhrubamalakar@yahoo.com (D. Malakar).

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Adipose tissue derived MSCs can be easily differentiated into osteoblasts, chondrocytes, lipocytes, sarcoblasts and other cell types (Zuk et al., 2001). The ADSCs have been isolated from human (Ramasamy et al., 2008), mice (Yoshimura et al., 2007), rat (Nishida et al., 2005), bovine (Bosnakovski et al., 2006), dog (Martinello et al., 2011), but very few studies have been reported in goats.

Somatic cell nuclear transfer (SCNT) is the most efficient and viable technique to propagate highly valuable endangered and extinct species (Lanza et al., 2000). Micromanipulator was routinely used to produce somatic cell cloned embryos in animals (Lagutina et al., 2007). However, the zona-free handmade cloning (HMC) technology is a preferred choice, with a higher success rate in producing cloned offspring in different species viz., horses (Lagutina et al., 2007), cattle (Tecirlioglu et al., 2005; Vajta et al., 2005), pig (Du et al., 2005) and buffalo (Shah et al., 2008). Handmade cloning (HMC) technique in goat is very significant on two accounts. First, handmade cloning is a simple and inexpensive technique. Second, dairy goats are ideal for production of many recombinant therapeutic proteins for application in human being and animals. In fact the first drug called ATryn, a human antithrombin protein was derived from a genetically engineered goat. The developmental potential of SCNT embryos has been demonstrated by using mammary gland cells (Wilmut et al., 1997), cumulus and granulosa cells (Wakayama and Yanagimachi, 1999; Wells et al., 2003) as donor cells. In goat, micromanipulator based cloning using adult granulose cells and fetal fibroblast cells have been reported by Keefer et al. (2002). Successful cloning has also been reported in goat by using handmade cloning technique with cumulus cells, fetal fibroblast cells (Akshey et al., 2010), putative embryonic stem cell, and lymphocytes as somatic donor cell (Dutta et al., 2011). But goat mesenchymal stem cell as donor cell for handmade clone embryo production has not yet been investigated. Therefore, the objective of the present study was to culture, propagate, and characterize the adipose tissue derived mesenchymal stem cells (ADSCs). The cultured ADSCs were selected as donor cells to evaluate the developmental competency of handmade cloned goat embryos. The present research finding could be of significance for future studies on assistant reproductive technology and therapeutic cloning for treatment of many degenerative diseases.

2. Materials and methods

2.1. Chemicals

Unless otherwise stated, all chemicals and reagents used in the present study were obtained from Sigma–Aldrich Chemical Company (Spruce Street, St. Louis, MO, USA). However, gentamycin (Cat. no. SV30080.01), DMEM/F12 (Cat. no. SH30023.01), fetal bovine serum (FBS, Cat. no. SH30396.02), Hanks basal salt solution (HBSS, Cat. no. SH300015.03) and bovine serum albumin (BSA, Cat. no. SH30574.02) were purchased from Hyclone company (South Logan, Utah-84321). The primary and secondary antibodies used in the present study were purchased from Santa Cruz Biotechnology company (Finnell Street, Dallas, TX, USA).

2.2. Isolation and culture of goat ADSCs

Adipose tissue was collected from flank region of a slaughtered goat in a nearby abattoir and transferred to laboratory in HBSS containing gentamycin (1%, V/V) at 4 °C with utmost sterile environment. Isolation of ADSCs was performed according to the method for rats (Niyaz et al., 2012), with some minor modifications. Briefly, fat tissue was minced in HBSS into very small pieces (1 mm in thickness) followed by three repeated washings. The minced tissues were incubated in digestion medium containing DMEM/F12 and 1% Collagenase Type I enzyme (Cat. no. C5894) for 2 h under standard culture condition with regular interval of manual shaking. The disaggregated tissues were filtered out to remove undigested fat tissues in a 41 μm filter paper (Millipore company, Billerica, 290 Concord Road, USA, Cat. no. NY4102500). To prevent the activity of Collagenase Type I enzyme the resuspended portions were incubated with same volume of growing medium (DMEM/F12 containing 10% fetal bovine serum) for 5 min under standard culture condition. The cell suspension was then centrifuged at $400 \times g$ for 10 min and the pellet was resuspended in the same volume of erythrocytes lysis buffer, containing 160 mM/l NH_4Cl (Stem Cell Technologies Inc., 570 West Seventh Avenue, Vancouver, Canada, Cat. no. 07850) in HBSS and incubated for 15 min at room temperature. After centrifugation, the supernatant was discarded out, and the cell pellet was resuspended at a density of 1×10^6 cells/cm^2 in growing medium. At 80% confluence, cells were trypsinized and sub cultured for further purposes.

Table 1
List of antibodies used during immunostaining.

Name of antibody	Synonym	Catalogue no.
CD29 (N-20)	Integrin β1	sc-6622
CD34(C-18)		sc-7045
CD44(T-20)	HCAM	sc-31043
CD90 (V-16)	Thy-1	sc-31244
CD166 (H-108)(	ALCAM	sc-25624
Cytokeratin 8 (H-40)		sc-134484
IgG-FITC		sc-2342

2.3. Cryopreservation and growth curve of ADSCs

Goat ADSCs at different passage numbers with 80% confluence are selected for cryopreservation. The growing medium from confluent monolayer culture was discarded, followed by rinsing with DPBS, and 0.25% trypsin–EDTA (Cat. no. T4049) was added to disaggregate the attached cells. The cells were further pelleted through centrifugation at $400 \times g$ for 10 min. The supernatant was discarded, and the cells were resuspended in freezing medium containing DMEM/F12, 10% DMSO (Cat. no. D2650) and 20% FBS at 0.5×10^6 cells/ml at −80 °C for 24 h, and stocked in liquid nitrogen. The cryopreserved cells were quickly thawed at 37 °C water. Cells at passage 3 were seeded in 12 well plates at 1×10^6 cells/ml. On the very next day, ADSCs were harvested for cell counting. The same procedure was followed for the next 8 days to generate a growth curve.

2.4. Scanning electron microscopy (SEM) of ADSCs

Adipose tissue derived mesenchymal stem cells were fixed in 4% glutaraldehyde (Cat. no. G5882) in PBS. After three repeated washings in PBS, samples were post-fixed in 1% osmium tetroxide (Cat. no. 75632) in Sorensen's buffer for 1 h. The ADSCs were then dehydrated through increasing concentrations of ethanol. The dehydrated cells were immersed in 15 min changes of hexamethyldisilazane (HMDS, Cat. no. 379212) for 1 h and the HMDS was allowed to evaporate overnight at room temperature. The processed cells were then mounted on SEM stubs, followed by allowing to off-gas in vacuum desiccators for 1 h, and then sputter coated with gold. Samples were examined and digitally imaged by a Scanning Electron Microscope (Model name – Carl Zeiss Ag – EVO40).

2.5. Characterization of goat ADSCs

Adipose tissue derived mesenchymal stem cells were characterized through immunostaining and amplification adult stem cell specific markers. For immunocytochemistry, briefly the ADSCs at passage 3 were fixed and permeabilized in 4% paraformaldehyde (Cat. no. 158127) and 0.5% (V/V) Triton X-100 (Cat. no. 93443) in DPBS respectively, followed by incubating in 4% normal goat serum for 30 min. The cells were then incubated with primary antibodies (1:100) (Table 1) overnight at 4 °C. In the respective negative controls, the addition of the primary antibody

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