

Original Article

Human Fallopian tube as a novel source of multipotent stem cells with potential for islet neogenesis

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Abstract

Presence of stem cells in the female genital tract has been reported; however stem cell status of Fallopian tube remains unexplored. In the present study, we show for the first time an existence of stem cells in a Fallopian tube. A pure population of mesenchymal like cells was obtained from the Fallopian tube samples from patients undergoing hysterectomy. The immunocytochemistry of these cells revealed the presence of classical mesenchymal stem cell markers like smooth muscle actin, vimentin, nestin, desmin, CD44, CD90 and CD117. These Fallopian Tube derived Mesenchymal stem cells could be induced to differentiate into adipocytes, chondrocytes, osteocytes, neuronal and pancreatic lineage under the influence of lineage specific differentiation cocktails. Such documentation of multipotent stem cells in a Fallopian tube may be of significance for instant repair of the tract as and when necessary so as to assist uninterrupted transport of eggs for possible fertilization thus facilitating reproduction.

Key words; Fallopian tube, Mesenchymal stem cells, multipotent differentiation

Introduction

Today, we are on the edge of a potentially explosive era in stem cell science and medicine. The regenerative medicine has focused on stem cells as a potential alternative to complicated tissue/organ transplantation. Thus, stem cells are impending as a biological substitute due to their enormous proliferative potential to renew and maintain the tissues they

populate^[1,2,3].

However, their use in therapeutic purpose has associated with many challenges, limiting their general use^[4]. Bone marrow derived stem cells are the main source for adult stem cells; however their use is restricted due to increased susceptibility to viral and bacterial infection^[5,6]. Also, adult stem cells are non immuno-compatible

unless isolated from the same patient; whereas ES cells exhibit the high degree of tumor formation and ethical concerns surrounding its use in research/therapeutic purpose. Therefore, much attention is now focused on alternative sources of stem cells.

Presence of stem cells in reproductive organs such as ovary and testis has been documented [7, 8, 9] however the potential of Fallopian tube as a stem cell source remained unexplored. The epithelial cells isolation from lining of the inner surface of the Fallopian tube has been first described by Henriksen et. al. to establish a method to culture these cells as a model for more specific studies of their properties [10] however their stem cells status remains to be seen. Although, reproductive system and accessory organs of female genital tract are not considered as vital organs like heart, kidney etc. they are responsible for reproduction and continuation of species. Since reproductive population/individual is a target for 'Natural Selection' the accessory reproductive organs are likely to be of significance as they are involved in transport of gametes. Hence, we hypothesized that Fallopian tube might be a source of stem/progenitor cells to facilitate instant repair of the genital tract to assist reproduction.

Materials and Methods

Fallopian Tube collection and cell isolation: Fallopian tubes were obtained from women undergoing sterilization by bilateral salpingo-oophorectomy at Patki Research Center and Hospital, Kolhapur, India. The informed consent was obtained from all 26 women. The protocol for collection and isolation was approved by National Centre for Cell Science's Ethical Committee.

Fallopian tubes were collected in sterile phosphate buffer saline (Gibco, Invitrogen, Carlsbad, CA) containing antibiotic (2µg/ml ciprofloxacin and 2 µg/ml amphotericin B) and transported to the laboratory. This collected tissue was finely minced and

digested using 0.15% Collagenase type IV (Sigma-Aldrich, St. Louis, MO) for 20 to 25 minutes at 37°C with constant agitation. The digest was then centrifuge at 1500 rpm for 10 min and cell pellet was seeded in DMEM (Gibco, Invitrogen, Carlsbad, CA) medium containing 10% hUCBS and supplemented with penicillin (100 units/ml), streptomycin(100µg/ml).

Medium was changed every 48h and replenished with DMEM supplemented with 10% hUCBS. Cells were first passage after 8 days and then after every 4 days.

Characterization of isolated cells using immunocytochemistry and flow cytometry:

The cells from early (4 to 6) passages were used for the characterization studies. Cells were grown in monolayers on coverslips were fixed using 4% paraformaldehyde followed by permeabilization using 50% methanol for 5 min. These cells were then treated with 5% Bovine Serum Albumin (BSA) in PBS for 1h to block all non-specific binding sites. The cells were then exposed to primary non-labeled mouse anti-human antibodies viz. nestin, vimentin, desmin and smooth muscle actin (SMA) (1:100 dilutions) (Chemicon, Temecula, CA) for 12 h at 4°C, followed by respective secondary antibodies (Invitrogen, Carlsbad, CA) for 1 h at 37°C. The coverslips were mounted in mounting medium containing antifade (Vectashield, Vector Laboratory, Burlingame, CA) and 4',6-diamidino-2-phenylindole (DAPI). The slides were then viewed using confocal laser scanning microscope (Zeiss LSM510). DAPI (Invitrogen, Carlsbad, CA) was used for nuclei visualization.

The cells from the passage 4 to 6 were dislodged using 0.05% trypsin 0.02% EDTA in PBS and resuspended in DMEM. The cells were fixed in chilled 70% ethanol and incubated in mouse anti human FITC/PE conjugated antibodies against CD33, CD34, CD44, CD45, CD73, CD90 and CD117 (1:100 dil) for 1h on ice (all the antibodies

were purchased from Becton Dickinson, San Diego, CA). The cells were acquired using a flow cytometer laser 488 nm (Becton Dickinson, NJ) and data was analyzed using BD Cellquest Pro software.

Differentiation Studies:

Induction of adipogenic, chondrogenic, osteogenic neuronal and pancreatic lineage: Human Fallopian tube mesenchymal stem cells (FTMSCs) at passage 3 were fed with alternate cycle of adipogenic induction medium (PT-3102B Cambrex, Walkersville, MD) and adipogenic maintenance medium (PT-3102A Cambrex, Walkersville, MD), adipogenesis was induced as per the manufactures instruction. Adipogenesis was confirmed using Oil Red O staining.

For chondrogenic, osteogenic and neuronal differentiation, 3×10^3 FTMSCs/cm² were plated onto tissue culture flasks and cells were allowed to adhere to culture surface for 24 h at 37°C. Chondrogenic, osteogenic and neuronal lineages were induced by replacing the growth medium (DMEM) with chondrogenic, osteogenic and neuronal differentiation bullet kit (PT-3003, PT-3002 and CC-3229 respectively Cambrex, Walkersville, MD) respectively as per manufacture's instructions. Chondrogenesis was confirmed using Safranin-O staining; osteogenesis by staining with Alizarin Red S while neuronal differentiation was confirmed by immunostaining with neuron specific markers Map2, NeuN (Chemicon, Temecula, CA).

The FTMSCs on reaching 80% confluency were seeded in serum free medium (SFM) [DMEM; insulin, transferin and selenium (ITS)] for 72h at 37°C. By day 3 hFTMSCs start forming cell aggregates. On day 4 SFM was supplemented with 0.3 mM taurine. On day 10 these ILCs were induced with a mixture of 100 mM nicotinamide, 3mM taurine and 100 nM glucagon like peptide 1 (GLP1). The floating islets were collected and characterized by incubating with 10µL

zinc-finger specific stain called diphenylthiocarbazon (DTZ) stain for 1 h at 37°C and viewed under inverted phase contrast microscope (Olympus IX 70, Tokyo, Japan). These ILCs were then stained with primary antibodies against human insulin and glucagon (Chemicon, Temecula, CA) and their specific secondary antibodies. The analysis was done using confocal laser microscope (Zeiss LSM510).

Results and Discussion

Isolation and expansion of hFTMSCs:

The results represent the summary of the data obtained using 27 Fallopian tubes. The culture protocol of hFTMSCs was optimized with different nutrient media. Digestion with 0.15% collagenase yielded 2×10^4 Cells per 5 cm long fallopian tube. The 100% confluency was reached after 4 to 5 days in culture. Optimum growth of hFTMSCs was obtained in the DMEM supplemented with 10% human umbilical cord blood serum (hUCBS) after screening different media (data not shown). Previously, our lab has shown the use of hUCBS to enhance the growth of stem/progenitor cell isolated from human bone marrow^[11] which was further confirmed by Shetty et. al.^[12]. Hence the hFTMSCs were supplemented by hUCBS instead of the fetal calf serum for better proliferation thus eliminating the use of xenoproteins for possible human usage.

Initial passages (0 to 4) showed a mixed population of epithelial and typical fibroblast like cells. The epithelial cell population decreased with increase in passage number. The epithelial cell population got eliminated after 5 to 6 passages and pure population of mesenchymal like cells was observed in the culture flask. During second week they showed typical slender fibroblast like cell phenotype (Fig 1A, B). This could be because of epithelial to mesenchymal transition^[13] commonly observed in development and regeneration.

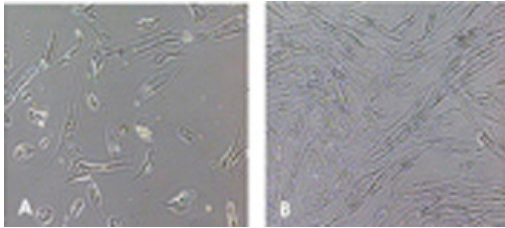
Figure 1

Fig.1. Mesenchymal like stem cells isolated human Fallopian tube showing mixed population of epithelial and fibroblast like cells (A). 24hrs after isolation while at passage 4 showing only fibroblast like cell morphology (B).

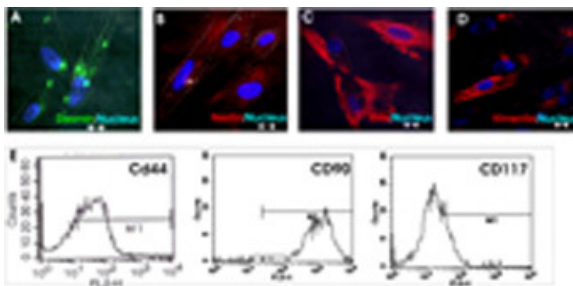
Figure 2

Fig.2. Characterization of Mesenchymal like stem cells isolated human Fallopian tube. Cells positive for surface markers: desmin (A), nestin (B), smooth muscle actin (SMA) (C), vimentin (D), and CD44, CD90, CD117 (E).

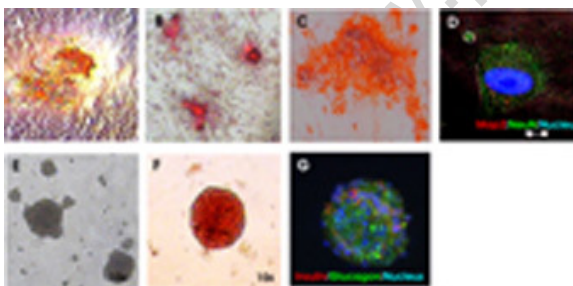
Figure 3

Fig.3. Multilineage differentiation potential of human Fallopian tube mesenchymal stem cells. Adipocytes (A) showing Oil RedO positive intracytoplasmic lipid droplets. Chondrocytes (B) showing Safranin positive proteoglycan matrix. Osteocytes (C) showing Alizarin red S positive calcium phosphate depositions. Neuronal lineage (D) showing Map2 (red) and NeuN (green). Pancreatic lineage showing islet like cluster (ILCs) phase contrast image (E). DTZ positive ILCs

(F) and insulin (red) and glucagone (green) positive ILCs.

Immunocytochemistry and Flow cytometry analysis:

The immunofluorescence study for specific cell surface markers clearly indicated that isolated human FTMSCs were positive for the mesenchymal markers viz. desmin (Fig. 2A), nestin (Fig. 2B), smooth muscle actin (SMA) (Fig. 2C), vimentin (Fig. 2C) and surface markers CD44, CD90, CD117 (Fig. 2E) and negative for CD 33, CD34, CD45, CD73 confirming their identity as MSCs. Presence or absence of certain markers on the cultured hFTMSCs is possible, as at present no known markers are specific for the adult stem cells [14, 15, 16, 17, 18].

Due to lack of specific known cell markers, stem cells are often phenotypically characterized by their lineage specific differentiation capacity. Therefore, we studied the potential of hFTMSCs to differentiate into ectodermal, mesodermal and endodermal lineage.

Multilineage differentiation potential of hFTMSCs:

Incubation of hFTMSCs in adipogenic induction medium led to differentiation into adipocytes like cells almost after 21 days. This adipocytic induction of hFTMSCs was seen by the change in morphology of cells from spindle-shaped to round to oval shaped cells, and by the appearance of numerous large, round intracytoplasmic lipid droplets. These lipid droplets were stained positive by Oil Red O (Fig 3A) confirming their adipocytes phenotype.

Human FTMSCs differentiated into chondrogenic cell lineage after 3 weeks of incubation in chondrogenic medium. Chondrogenic phenotype in induced hFTMSCs was seen by the changes in cell morphology, from spindle shaped to larger round cell aggregates, and by the accumulation of sulfated proteoglycans

which are present in cartilage. These proteoglycans in the matrix stained positive with Safranin-O (Fig. 3B) indicating their chondrocyte status.

Exposure to osteogenic differentiation medium for 3 weeks hFTMSCs showed changes in cell morphology, from fibroblast like to cuboidal shape with progressive differentiation and mineralized. Calcium phosphate mineralization stained positive by Alizarin red S stain indicating direct evidence of calcium deposits as amorphous accumulation between cells (Fig 3C) after 3rd week of osteogenic induction.

When exposed to neuronal differentiation cocktail for 21 days, hFTMSCs changed their morphology to neuronal like cell. These cells stained positive for neuronal markers Map2 and NeuN (Fig.3D), disclosing their identity as neurons.

In addition to this, our studies also demonstrate the potential of hFTMSCs to differentiate into islet lineage (Fig.3E). Our protocol of islet differentiation involved time dependant sequential addition of ITS, taurin, nicotinamide and GLP1 in a serum free medium leading to the progressive cell clustering from day two onwards which led to typical islets like clusters (ILCs) formation at the end of day 10 (Fig 3E). The differentiation medium cocktail has been standardized and reported previously^[19].

After 10 days of induction these mature ILCs stained positive for the islet-specific DTZ stain (Fig. 3F), which is known to selectively stain pancreatic beta cells because of their high zinc content, non-islet tissue remained unstained. These clusters were also found positive for insulin, glucagon (Fig. 3G) by immunocytochemistry. Pfeifer and Chegini have previously shown the presence of IGF-I, IGF-IR, and IGF-BPs 1-4 in the human Fallopian tube during various reproductive stages^[20]. This finding strengthens our data

indicating presence of requisite niche and hidden potential of hFTMSCs to differentiate into the pancreatic lineage.

Thus in the present study we have demonstrated for the first time presence of mesenchymal stem cell population in the Fallopian tube exhibiting trilineage differentiation potential confirming our hypothesis. Our findings further provide yet another source of multipotent progenitors for possible autologous transplantation in addition to bone marrow. The documentation of multipotent stem cells in a Fallopian tube may be of significance for instant restoration of the tract on damage to assure uninterrupted transport of eggs for possible fertilization facilitating reproduction.

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