



An efficient method of generating skin fibroblast cells for cell banking

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ABSTRACT

An optimum cell culture medium is used to generate fibroblast cells for cell banking. Here, we describe a method for obtaining higher number of pure fibroblast cells in much shorter duration from skin tissue explants. Two pronged strategy was used, first two different culture media were selected, one for preferential generation of fibroblast cells in primary culture (Human fibroblast specific media- HifibroXL) and another for their faster multiplication in secondary culture (DMEM + Ham's F12). Secondly, tissue explants were cultured not only once but up to six times increasing the generation of primary cells *per se*. This method initially standardized with buffalo (*Bubalus bubalis*) skin tissue explants works efficiently with camel (*Camelus bactrianus*) and horse (*Equus caballus*). Modified cell culture method increases the efficiency of establishing fibroblast cell banks by reducing the cost both in terms of consumables and human effort. It is well suited for today's fast-paced conservation laboratories.

Key words: Buffalo, Epithelial cells, Explant culture, Genebank, Skin fibroblasts

Fibroblasts are the most prevalent cells in dermis, an inner thick layer of skin (Tracy *et al.* 2016). Fibroblast cell lines establishment and their use in investigating the gene or protein functions of the cell is substantially contributing towards the conservation and utilization of genetic resources. Cryopreservation of somatic cells creates an interesting option for preservation of endangered animals, breeds or species both in case of wild and domestic animals (Wu 1999). The development of somatic cell cloning technology and the establishment of somatic cell bank for the purpose of recovering endangered mammalian breeds and species is especially important. Moreover, *in vitro* culture of fibroblast cells provides an excellent model system to study the molecular mechanisms regulating fibroblast function in normal and disease tissues, many aspects of cell physiology, particularly those related to skin biology and reprogramming/induced pluripotency studies. In addition, these cells offer great promise for producing site-specific genetically modified animals. For the success of such studies, establishment of species, tissue or disease-specific fibroblast cell lines with high rate of proliferation, longevity, genetic stability, and the potential for *in vitro* genetic manipulation is required (Singh *et al.* 2011).

Fibroblasts have been successfully cultured *in vitro* from several species (Singh and Sharma 2011, Liu *et al.* 2014, Mehrabani *et al.* 2014). Fibroblasts from ear tissue can be obtained without risk to the donor animal's health and are practical to harvest and sub-culture as compared to other

cells (Hosseini *et al.* 2008). However, fibroblasts are also a finite resource due to their limited life span. This limitation can be overcome by increasing the number of fibroblasts obtained initially from the tissue explants (Huschtscha *et al.* 2012). At present, fibroblasts are obtained from skin tissue explants by using a single culture medium for the primary and secondary culture. In this brief communication, we describe a method to obtain higher number of primary fibroblasts from a skin tissue sample that can be used for subsequent generation of large number of secondary cells. In addition, fibroblast cells become free of epithelial cells at a faster rate. Together, it leads to the reduction in time and efforts required for obtaining the cells that can be used in experimentation or for conservation. It becomes even more pertinent for generating viable cryo-preserved cell lines from very small amount of biopsy material that will be available from animal of endangered species.

MATERIALS AND METHODS

Sample collection: Buffalo ear tissue was used as the experimental material. Tissue samples were slaughter house by-products and collected from authorized abattoir at Delhi, India. All the cell culture media and chemicals were purchased from Sigma, USA unless otherwise indicated. Human fibroblast specific media- HifibroXL was procured from HiMedia, India. Ear tissue pieces were punched from tissue and transported to the lab in a tube containing DMEM/ Ham's F-12 (Dulbecco's Modified Eagle's Medium/ Ham's Nutrient Mixture F12 1:1) with antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin) at 4°C.

Tissue explants culture: Two commercially available media, viz. Human fibroblast specific media (HiFibroXL)

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and DMEM/Ham's F12 were tested for outgrowth of fibroblasts from buffalo tissue explants. Tissue was chopped into 1–3 mm² pieces after removing hair, epidermis and fat layer. Tissue explants were incubated in pre-warmed media having 20% FBS (Fetal bovine serum) and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin) at 37°C, 5% CO₂ in a humidified incubator. Next day, 3 ml of additional media was added to the petriplates without disturbing the explants. Explants were incubated undisturbed for 6–7 days (Singh and Sharma 2011). Media was replaced every fourth day to remove cell debris. Explants were observed for migration of cells under inverted microscope every alternate day after one week.

Harvesting of cells: The skin explants were transferred aseptically to a new petri dish for generating primary cells for the second time when sufficient cells had migrated from the explants producing an outgrowth of about 20 mm. Care was taken to invert the explants and steps as described earlier for the adherence of explants and media addition were followed. Meanwhile, the cell monolayer in the original dish was harvested using 0.25% trypsin-EDTA (Singh and Sharma 2011). Cell pellet was re-suspended in 1 ml of respective growth medium. Cell count and viability was established with trypan blue dye exclusion method (Freshney 2011). Epithelial cells were detected under the microscope as well as by RT-PCR (Liu *et al.* 2014). During serial passaging of buffalo fibroblasts, apart from FBS (10%) and cell seeding density (80,000 cells/flask); rest of the procedure was same as described above.

Selection of cell culture media: Fibroblasts of the 4th passage cells were used for assessing the growth potential of cell culture media for the secondary fibroblast culture. Cells were seeded in 25 cm² cell culture flasks at a density of 8×10^4 cells per flask. These were cultured for 8 days at 37°C and 5% CO₂ in a humidified incubator and cells were counted every day (3 flasks each time). The mean cell numbers at each time point were adopted to plot a growth curve and to calculate the population doubling time (PDT). The multiplication rate (r) and population doubling time (PDT) was determined along the exponential phase of the cell growth ($r = 3.32 (\log N_H - \log N_i) / (t_2 - t_1)$, PDT = $1/r$; N_H is number of cells harvested, N_i is number of cells seeded, t_2 is hours during harvest and t_1 is hours at initial time).

The mean and SE of counted cells in growth curve analysis were compared using independent sample t-test (SPSS for Windows, version 11.5, SPSS Inc, Chicago, Illinois). Values with $P \leq 0.05$ were considered significantly different.

RESULTS AND DISCUSSION

At about two weeks after the tissue explants adhered to the flasks, cells were observed to be sprouting from the margins of these tissue pieces in both the media (Fig. 1). This was consistent with the time duration reported in literature for fibroblast emergence in various species (Pei *et al.* 2015). The principal concern during primary culture of skin tissue explants is to obtain the fibroblast cells instead

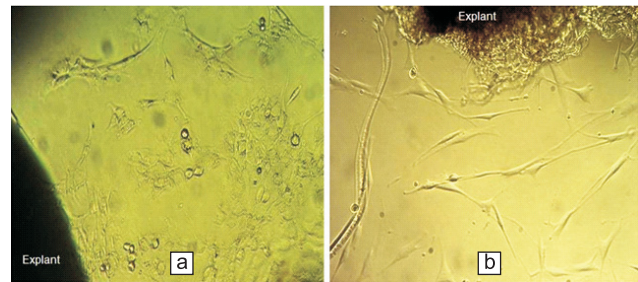


Fig. 1. Morphological difference in ear tissue explant outgrowth at day 14 of culture in (a) DMEM/Ham's F12 media (b) Human fibroblast specific media ($\times 40$ magnification).

of other possible cell types such as epithelial or osteocytes. Morphological observations indicated that the first outgrowth from the explants was heterogeneous. Outgrowth of fibroblast as well as epithelial cells was recorded from the adhered tissues in both the media. However, epithelial cells were negligible and concomitantly more fibroblast cells were growing in fibroblast specific media (HiFibroXL) as compared to DMEM + Ham's F12 (Fig. 1).

Fibroblasts get trypsinized as well as get adhered more easily and readily than the epithelial cells (Liu *et al.* 2014). They have higher growth potential and with serial passaging gradually overwhelm their epithelial counterparts (Hui *et al.* 2012). Because of these characteristics, a culture of pure fibroblasts is obtained after four to five passages (Mehrabani *et al.* 2016). It has been reported in literature that biological characteristics, especially those determining hereditary traits might be undermined due to repeated trypsinization (Hui *et al.* 2012). So, a minimum number of passages (<4) for fibroblast cells are recommended for preserving a species for posterity (Liu *et al.* 2014). In the present study, primary culture in the HiFibroXL substantially reduced the growth of epithelial cells from the skin tissue explants. Epithelial free fibroblast culture was established after the first passage itself (Fig. 2). As a result, time and material required for obtaining the fibroblast cells were also reduced.

Once the sufficient fibroblasts had migrated from the tissue explants, the explants were transferred into a new petri plates for re-culture. Higher proportion of the fibroblast cells were observed in the HiFibroXL, thus, it was selected for culturing of tissue explants for multiple times. Number of explants adhered to the plate was recorded as fibroblast

Table 1. Effect of reseeding on tissue explant culture

Re-seeding	Number of adhered explants* (N=3)	Percentage decrease in anchored explants	Days taken for initial cell growth (N=3)
1	29 ± 0.58	3.33	13.67 ± 0.88
2	26.3 ± 0.88	12.22	6.0 ± 0.58
3	23 ± 0.58	23.33	4.33 ± 0.33
4	16.3 ± 0.88	45.56	3.0 ± 0.58
5	13.7 ± 0.88	54.44	2.67 ± 0.33
6	11 ± 0.58	63.33	3.0 ± 0.00

*Thirty explants were reseeded in one culture dish.

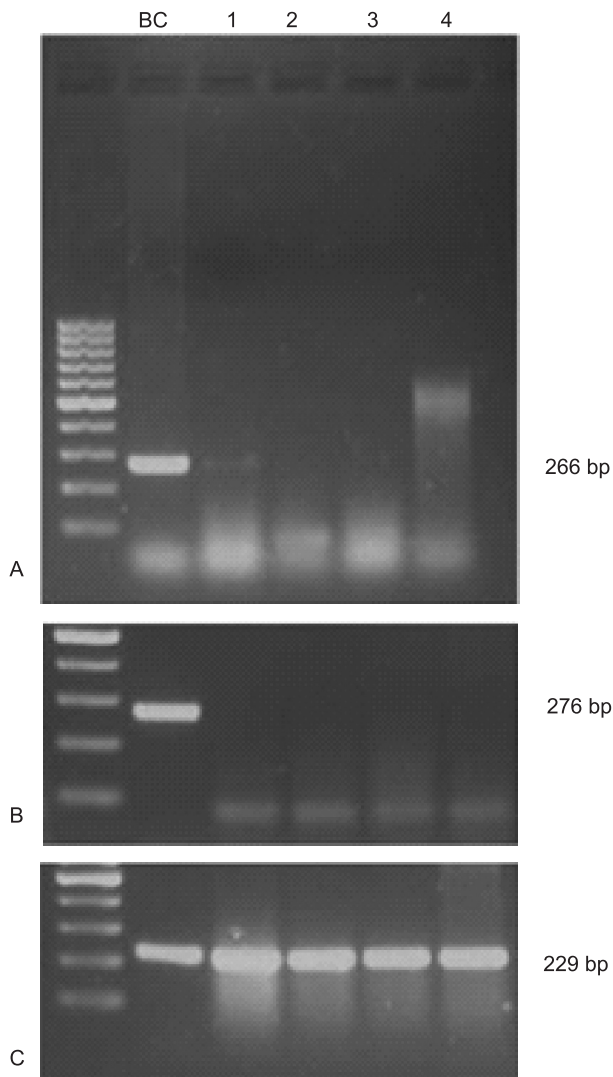


Fig. 2. Detection of epithelial cells (A) and osteocytes (B). Markers of epithelial (Cytokeratin 19) and osteocytes (Osteopontin) were detected by RT-PCR. BC is Buffalo control DNA, lanes 1–4 depicts passage 1 to 4 cells; C is GAPDH (Glyceraldehyde-3-Phosphate Dehydrogenase) amplification.

growth was observed around the adhered explants only (Singh and Sharma 2011). The number of adhered explants decreased, whereas, cell outgrowth became faster with every re-seeding (Table 1). Faster cell growth may be attributed to the fact that explants initially took time for adjusting in new environment of *in vitro* culture which became less in the later stages.

According to standard protocols, skin explants are used up to two transfers in case of human explants and generally used once before discarding the explanted tissue (Freshney 2011). In the present study, explants were transferred up to 6 times. Experiment was terminated only after that when the number of explants anchoring to the tissue culture plate reduced to less than 40% (Table 1). Culture of the same skin tissue explants for multiple times will be important for endangered species where sample availability is limited, or even for species where obtaining the sample is itself a difficult task. It has been reported that human explants can

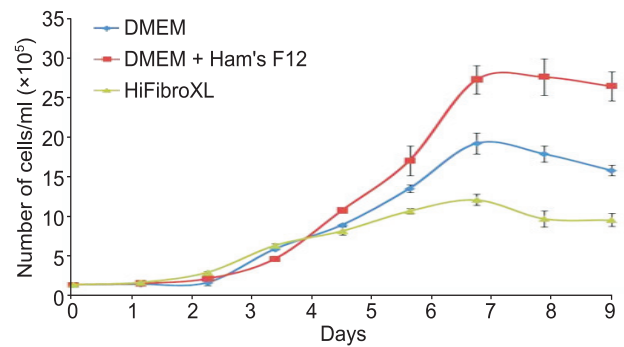


Fig. 3. Growth curve of fibroblast cells (Mean \pm SE of three observations) in different culture media.

be transferred sequentially up to 80 times, if required, at which point the explants appear to be completely depleted of fibroblast (Huschtscha *et al.* 2012). Hence, the number of early passage cells can be increased to many folds by above mentioned protocol.

Once the superiority of fibroblast specific media (HiFibroXL) was established for the generation of primary skin fibroblasts, its potential was further tested for supporting growth of secondary fibroblast cells. The main objective here was to achieve the fastest multiplication of cells to obtain maximum freezable cells. Good cell health and sufficient cell density are two important factors to be ensured. In addition to the two media used for primary culture (DMEM/Ham's, HiFibroXL), another commonly used media for fibroblast culture, DMEM (Dulbecco's Modified Eagle's Medium) was compared for supporting growth of fibroblast cells in secondary culture. Best medium was selected on the basis of morphology, confluence, cell count and viability of fibroblasts. On the basis of these criteria, DMEM/Ham's F-12 was the best media followed by the DMEM.

The proliferation potential of fibroblast cells in the different media was epitomized by the growth curve. The results showed typical mammalian growth curve which was similar but not identical. It can be seen from the growth curve (Fig. 3) that irrespective of the media, maximum number of fibroblast cells could be attained on the day six of the culture. However, media differed significantly ($P \leq 0.05$) with respect to the maximum number of fibroblast cells, being highest in DMEM/Ham's F-12 followed by DMEM and the lowest in the fibroblast specific media.

Total cell count in the HiFibroXL media ($11.85 \pm 0.74 \times 10^5/\text{ml}$) was less than half of that observed in the DMEM/Ham's F-12 ($27.50 \pm 1.84 \times 10^5/\text{ml}$) on day 6. It was quite perplexing observation as the trend was opposite during initial phase of the growth. Number of cells was higher in the HiFibroXL media as compared to the DMEM/Ham's F-12 on the second day ($2.4 \pm 0.09 \times 10^5/\text{ml}$ and $1.58 \pm 0.06 \times 10^5/\text{ml}$ cells respectively). Moreover, lag phase of growth corresponding to the adaptation and recovery period of the cells against trypsin damage was less in the HiFibroXL media (Fig. 3). This confusion was resolved by the morphological observations made under the microscope.

The fibroblast cells showed typical fusiform morphology with centrally located oval nuclei in all the three media. The cells had fibroblastic characteristics with turgor vital cytoplasm, fibroblast-like radiating, and flame-like migrating patterns (Singh *et al.* 2011). However, the cells in HiFibroXL media depicted maximum confluence at any day and were the first to cover the bottom surface of tissue culture flask (Fig. 4).

Confluence is commonly used to estimate adherent cell number in a culture dish or flask, referring to the proportion of the surface, which is covered by cells. It may be because of the presence of additional growth factors and/or hormones in the fibroblast specific medium which stimulated synthesis of elongated and larger cytoplasmic cell extensions. Large cells in turn covered the tissue culture surface much faster than the smaller cells. This could have also resulted in the phenomenon of contact inhibition causing reduced cell multiplication (Hui *et al.* 2012). Thus, in fibroblast specific media cells had shorter lag phase, occupied flask surface at a faster rate and appeared much healthier but had reduced cell number.

Morphological observations were well supported by the difference in the cell multiplication rate (r) and population doubling time (PDT) in the three media. Highest rate of proliferation was observed in DMEM/Ham's F-12 followed by DMEM and the least in the human fibroblast specific

medium ($r = 0.042 > 0.03 > 0.023$ and $PDT = 23.67 \text{ h} < 32.57 \text{ h} < 42.52 \text{ h}$). Optimum conditions including suitable culture medium describe PDT varying between 22–25 h for highly proliferative fibroblast cells of different species (Singh *et al.* 2011, Hui *et al.* 2012, Mehrabani *et al.* 2016). It is rational to conclude that DMEM/Ham's F-12 media is a better choice for secondary fibroblast passages. DMEM/Ham's F-12 media is extremely nutritious and supports growth of a wide variety of cells including certain epithelial, endothelial and granulosa cells (Yi *et al.* 2012). Use of fibroblast specific media for primary and DMEM/Ham's F-12 media for secondary culture has been optimized for buffalo fibroblast cells. We validated applicability of this approach for two more species, camel (*Camelus bactrianus*) and horse (*Equus caballus*) fibroblast cell lines (unpublished data). Thus, the presented culture strategy has broader applicability under standardized *in vitro* conditions. The newer approach described in this communication might increase the efficiency of generating fibroblast cryobanks both quantitatively and qualitatively. Further experiments including establishment of hereditary stability and transfection efficiency of the fibroblasts generated by explant re-culture needs to be established.

In summary, the method describes uses two different media for primary and secondary culture of skin fibroblasts. Re-culturing of tissue explants in fibroblast specific media

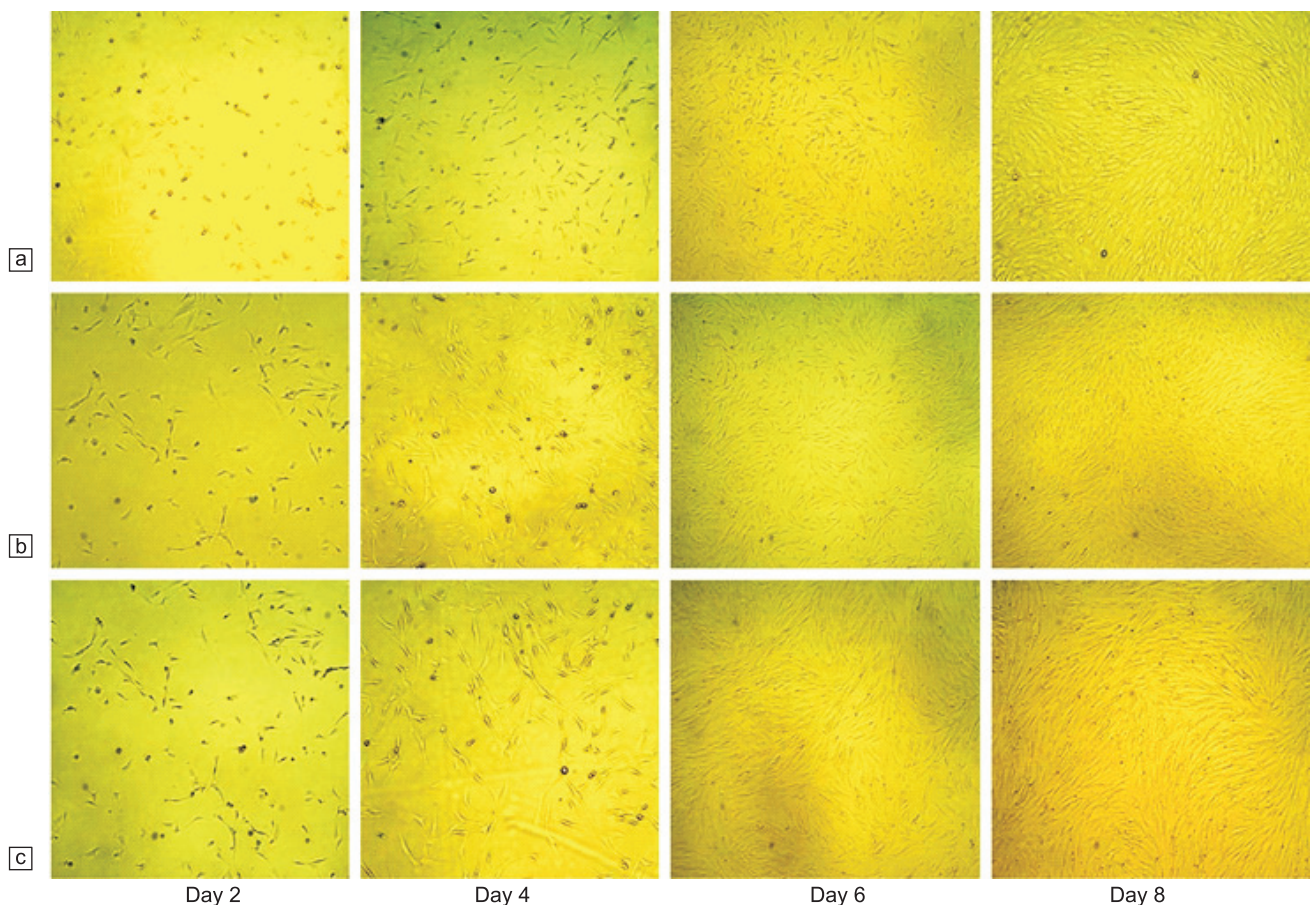


Fig. 4. Growth of secondary fibroblast cells in different media for a period of 8 days. (a) DMEM (b) DMEM/Ham's F12 (c) Human fibroblast specific media ($\times 10$ magnification).

provides a new platform to enhance the generation of primary fibroblast cells. This method leads to maximum utilization of the precious tissue sample which will make fibroblast cell cryopreservation more efficient and faster and can be a method of choice for the rapid creation of cell banks.

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