IBBJ

Summer 2017, Vol 3, No 3

Original Article

Spontaneous Mesenchymal to Epithelial Like Tissue Transition (MET) in a Long Term Human Skin Culture

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Submitted 24 Feb 2017; Accepted 31 Mar 2017; Published 13 Jun 2017

In an attempt to isolate multipotent stem cells from foreskin in a long-term culture, we encountered an interesting phenomenon which was the conversion of the fibroblast dominant condition to epithelial-like tissue formation. However, the basic mechanism(s) which may be involved in this conversion is not clear. This study was designed to evaluate the cells protein secretion activity and examine the role of oxidant/antioxidant capacity in this mesenchymal to epithelial cells transition (MET)-like phenomenon. The explanted tissues were obtained by spread out of the small sized foreskin derived tissue onto the cell culture dishes upona 40 –day incubation period in DMEM. After this period, the supernatant was collected and the amounts of glucose, total proteins, antioxidant capacity and protein profiles were determined and compared to the baseline medium. Also, routine hematoxylin and eosin staining was performed. Fibroblasts and uncharacterized fibers emerged from beneath of the specimen during the first week, and gradually overgrew within the first month. Surprisingly, these cells began to disappear around day 30 while epithelial-like cells turned out to be the major cells in cell culture dishes. Ultimately on day 40, the epithelial-like cells appeared. Total protein concentration was 1.44 mg/dl in the old medium versus 0.97 mg/dl in the baseline medium. The concentrations of glucose were 1.6 and 119.2 mg/dl for the old and the baseline medium, respectively. The antioxidant activity of the old medium was 176.29 µM, in comparison with the baseline medium 96.63 µM. There were differences in protein patterns between the two media on SDS-PAGE. The density of some proteins with molecular weight of 8-89 kDa was higher in the old medium corresponding to 40-day culture. The generated data showed that MET can take place *in vitro* probably through secretion of some small to intermediated sized proteins in a redox favored microenvironment. This can be considered as a good model for *in vitro* study of MET in metastatic tumors.

Keywords: Dermal fibroblast, epithelial cell, foreskin culture

Fibroblasts as the prototype of mesenchymal cells have different roles in health and diseases. These ubiquitous cells produce the collagen fibers and secrete matrix related proteins to establish three

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dimensional scaffolds for cells in most tissues. In wound healing process, these cells play some pivotal roles through secretion of a dozen of cytokines and growth factors. As cancer associated fibroblasts, these motile cells are recruited by cancerous cells to promote tumors growth and progression through angiogenesis induction. Also, there are some evidences that mesenchymal cells can be transformed into epithelial cells in some carcinomas (1). Other studies showed that epithelial to mesenchymal cells transition (EMT) are required for carcinoma type tumor metastasis and the reverse phenomenon i.e. mesenchymal to epithelial transition (MET) is a necessary phenomenon in establishment of metastatic tumor lesions (2). These phenomena may be related to oxidative stress. At least for EMT phenomenon the role of this type of cell stressor is clear (3). Moreover, skin fibroblasts because of their easy accessibility and convenient *in vitro* culturing, are used as the main component of the human skin substitutions which are used in routine clinical setting. Inducible pluripotent stem cells (IPS) preparation is a fibroblast based approach which opened a promising horizon in regenerative medicine (4). In an attempt to isolate the multipotent stem cells from foreskin, we encountered an interesting phenomenon which was the conversion of the fibroblast dominant condition into epithelial-like tissue formation in a long-term culture. However, the basic mechanism(s) which may be involved in this conversion is not clear. It can be considered as a MET like phenomenon, and understanding the underlying mechanisms will open a new window on MET details in metastatic tumors, and probably will allow exploring some ways to interfere with tumors growth in their new home.

This study was designed to evaluate the cells protein secretion activity, and examine the possible role of oxidant/antioxidant capacity in this MET-like phenomenon.

Materials and methods

Fibroblasts isolation

Newborn human foreskins were prepared by surgery from children aged 3 months who underwent routine circumcision at Amirkola children's hospital, Babol, Iran. The skin samples were washed 5 times in PBS and non-enzymatic method was used for fibroblasts isolation. To this end, the foreskin was placed on two sterile plastic tissue culture dishes (Orange scientific cat: 4450100N) and minced to small pieces using scissors. The dishes containing samples and complete growth medium DMEM (PAA cat: E15–883) supplemented with FBS 10% (PAA cat: A 15-15) and penicillin/streptomycin 1% (PAA, cat: p11-010) were placed in a humidified 37 ^{o}C, 5% CO_2 incubator. The growth of fibroblasts was checked every 2-3 days under an inverted phase contrast microscope (5). After fibroblasts growth for 3 weeks, the remaining tissue pieces were removed and the cells monolayers were washed with sterile PBS and replaced with complete medium, and one of the dishes was incubated at 37 ^{o}C. After a 40 –day period, glucose, total protein, antioxidant capacity and protein profiles were determined in the cell culture supernatant, and compared to DMEM supplemented with antibiotics and FBS as baseline medium. Also the dishes were stained by routine hematoxylin and eosin method.

In another dish, after removing PBS, cells were detached with 0.25% trypsin–EDTA (Sigma: 5 9418C), centrifuged at 400 g for 5-7 min, the supernatant was removed carefully, and the cell pellet was transferred into a new flask for subculture.

Determination of glucose and protein levels in cell culture supernatant

The supernatant of the fibroblasts old culture was collected. Protein assay was carried out using Bradford method, while Benedict method was used for glucose levels determination. Complete medium was used as control.

Ferric reducing antioxidant power (FRAP) assay

FRAP as a simple and reliable colorimetric method was used for measuring the total antioxidant

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capacity (6). This method is based on the reduction of Fe^{3+} to Fe^{2+} by the antioxidants. Briefly, FRAP reagent contained 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine; Sigma), 20 mM $FeCl_3$, 6H2O plus 0.3 M sodium acetate buffer pH 3.6 (1:1:10). 50 µl sample was added to 1.5 ml FRAP reagent and incubated at 37 °C for 10 min. The absorbance of reaction mixture was measured at 593 nm. $FeSO_4$ 1 mM was used as the standard solution in serial dilution 1000, 500, 250, 125 µM, and calibration curve was prepared (7).

Sodium dodecyl sulphate- Polyacrylamide gel electrophoresis (SDS-PAGE)

Determination of protein levels from cell culture supernatant and complete medium was done by Bradford method and identical concentrations were used for electrophoresis. Protein electrophoresis analysis was carried out by 13.5% SDS-PAGE according to Laemmeli method (8). Briefly, the samples were mixed with loading buffer containing 20% glycerol, 0.004% bromophenol blue, 4% SDS, 10% 2-mercaptoethanol, and 0.125 M Tris–HCl, pH 6.8. The mixtures were heated in boiling water for 5 min and then were loaded into polyacrylamide gel. Electrophoresis was conducted in stacking and resolving gel, respectively with 80 and 180 V in a tank from Amersham company. Then Coomassie Blue G250 was used for gel staining (9).

Hematoxylin and eosin (H&E) staining

After harvesting cell culture supernatant, the adherent cells were stained with H&E based on routine protocol.

Results

Metabolically active status with high redox capacity in skin old culture

To grasp an overall view about the possible underlying mechanisms in the observed cell transformation, we assessed the glucose consumption and protein secretion as some basic biochemical markers for cells activity in general. We assumed that this cell transformation derived from oxidative stress. To examine this hypothesis, the total redox capacity of the harvested cell culture supernatant was determined by FRAP assay. As Table 1 shows, the cells highly consumed the glucose, and secreted proteins at a remarkable level. Surprisingly, harvested medium from the skin old culture exhibited a higher level of antioxidant capacity in comparison with fresh completed medium.

Transformation of fibroblast to epithelial like cells

In attempt to isolate stem cells from neonate foreskin through non-enzymatic method, we found that fibroblasts transformed to epithelial like cells. As shown in figures 1 and 2, initially fibroblasts migrated from explanted skin, and continued to grow around the skin tissue (Fig. 1A,3A), and then reached moderate (Fig. 1B, 3B) and high confluence (Fig. 1C, 1D, 2C) in cell culture plate. Then interestingly, after almost 30 days of tissue culturing, these cells started to transform into polygonal epithelial like cells, and ultimately on day 40, the epithelial like tissue appeared (Fig. 3D).

Proteins with molecular weights of 8-89 kDa in old skin culture

To examine the hypothesis that the observed cells transformation was mediated by some unknown protein, the SDS-PAGE protein migration pattern of the supernatant of the skin old culture was compared with 10% FBS supplemented cell culture medium.

Table 1. Determination of glucose, protein, antioxidant activity

	Glucose (mg/dl)	Protein (mg/dl)	Antioxidant (µM)
Control	119.2	0.97	96.63
Cell culture supernatant	1.6	1.45	176.3

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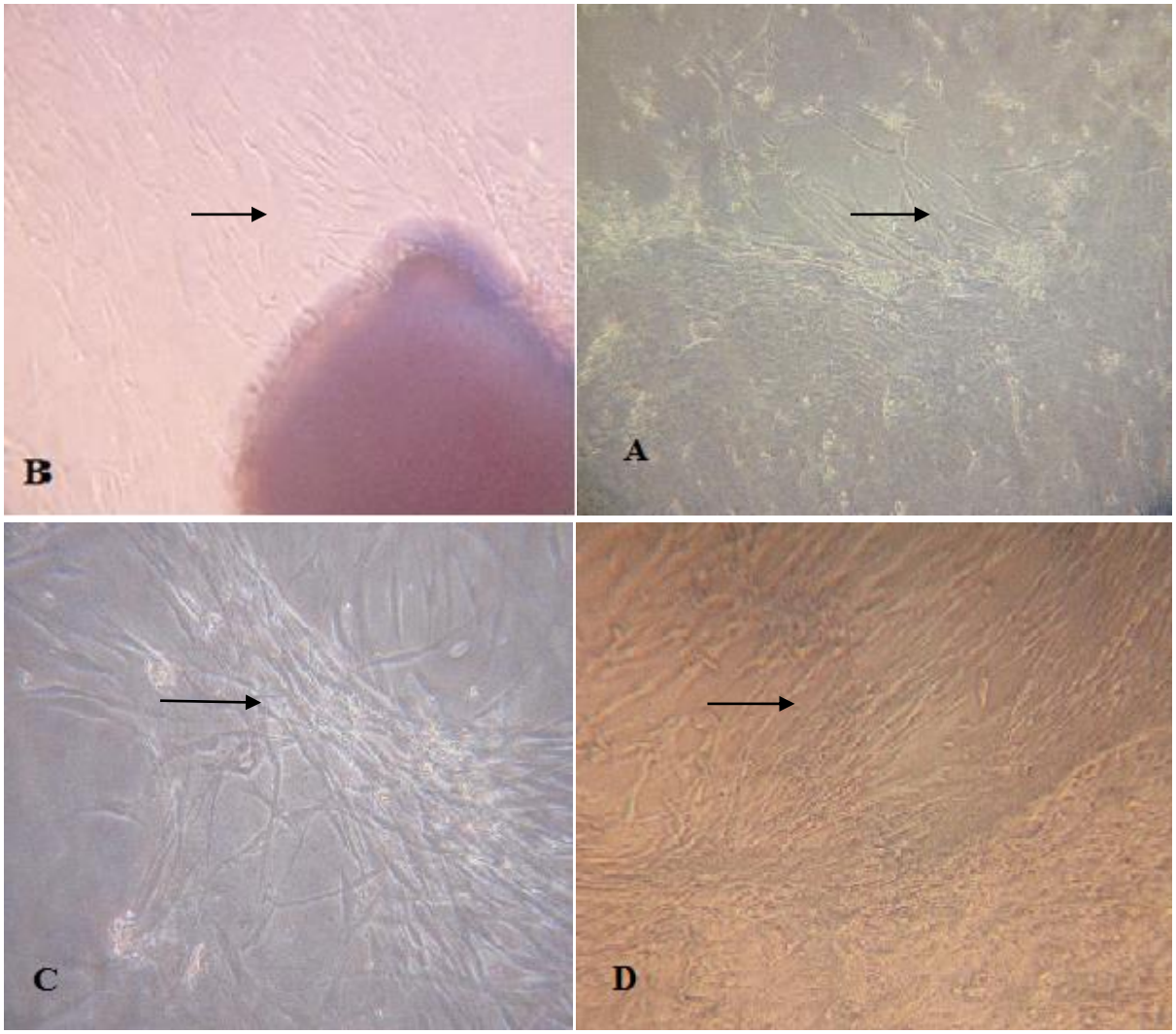

Figure 1. Fibroblast migration from explanted skin (10X). After one week fibroblasts initiated to exit from the tissue, and proliferated and increased their quantity in the plate.

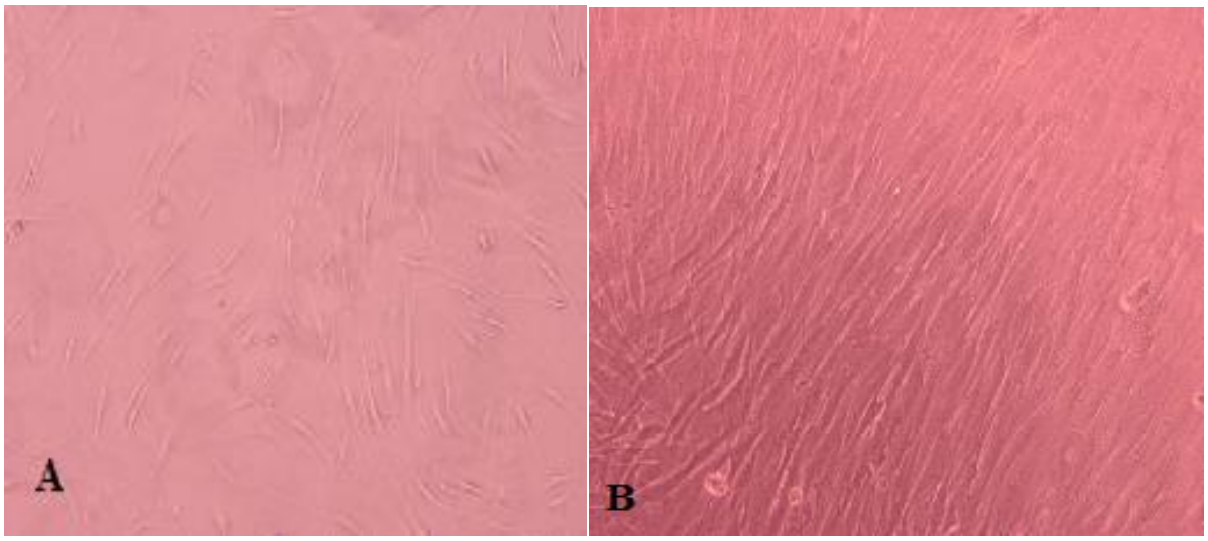

Figure 2. Microscopic appearance of fibroblasts in flask (10X). When fibroblasts proliferated and reached confluence in the plate, they were tripsinized and transferred into flask, the media of flask were changed every 3 days. A: morphology at low density; B: morphology at high density.

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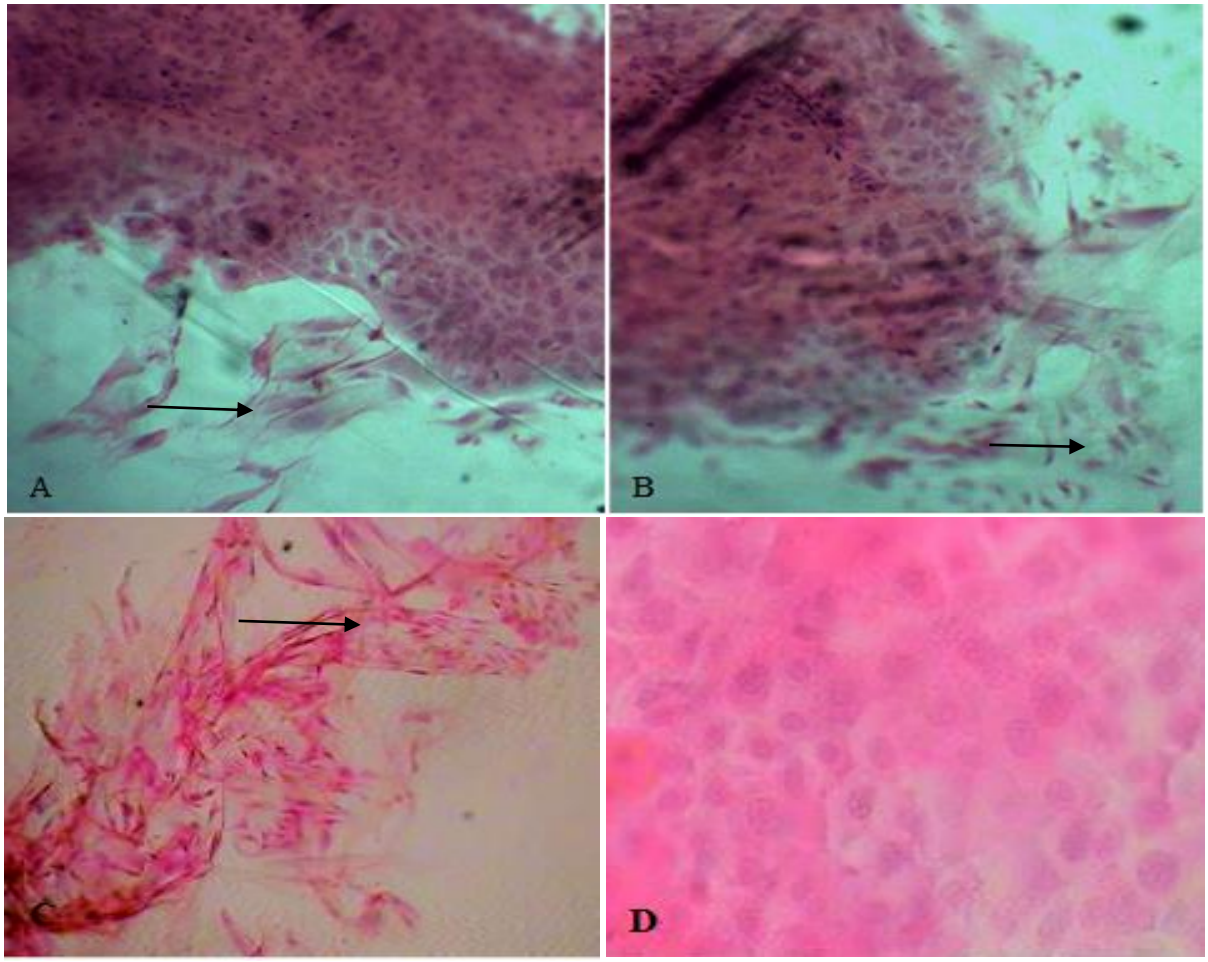

Figure 3. H&E staining of fibroblast and epithelial cells (20X). After almost 30 days of tissue culturing, fibroblasts initiated to transform into polygonal epithelial like tissue, and finally on day 40 the epithelial like tissue appeared. A, B: fibroblasts exit from tissue; C: fibroblasts; D: epithelial cells.

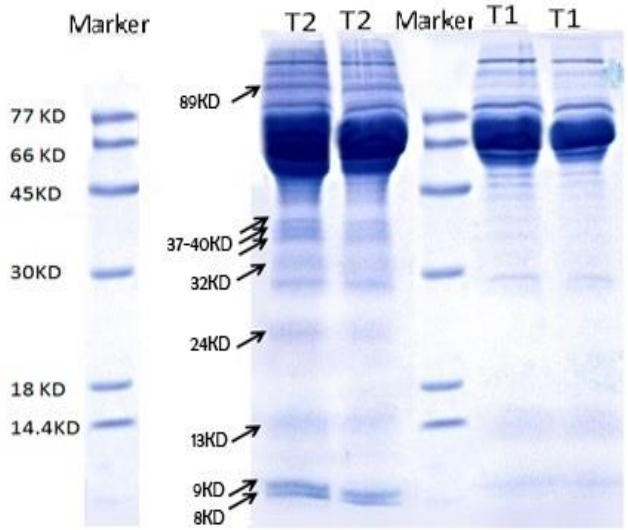

Figure 4. The protein profile of the culture supernatant prepared from the old skin culture. T2: supernatant of the skin old culture; T1: 10% FBS supplemented cell culture medium. Differences in migration of proteins with molecular weights of 8, 13, 24, 32, and 89 kDa were observed between supernatant of cell culture (T2) and medium (T1) (Fig. 4). So proteins with these molecular weights are much more abundant in cell culture than in 10% FBS supplemented medium. Two protein bands with small differences in size, nearly 8 kDa, were outstanding in skin old culture supernatant in comparison with fresh completed medium. Three protein bands in the range of 37-40 kDa were seen in the supernatant of cell culture (T2) but were not visible in T1.

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Discussion

Mesenchymal- epithelial transition is a biological process which plays some important roles in health and diseases. For instances, it is involved in decidualization which in turn is considered as a vital phenomenon in blastocyst implantation in uterus (10). Moreover, its function also seems to be prominent during nephrogenesis (1). Metastatic cancer cells also make benefit from this process to settle down properly in their new microenvironments (11). This process can also be induced *in vitro* by silencing of *SOX2*, a stemness gene (12), or by over expressing E-cadherin (13), and by some drugs like lapatinib induced mesenchymal-epithelial transition in squamous cell carcinoma cells (14). However, to our knowledge, we are the first group reporting the spontaneously *in vitro* occurrence of the MET like phenomenon. Although the evaluation of the epithelial cells specific markers such as cytokeratin or E-cadherin is necessary to provide more detail, but this study can be considered as a primary study to establish a new *in vitro* model of MET. Interestingly, during IPS production from mouse fibroblast, the MET was also observed by another group (15). To interpret this observation, we showed that the cells were active metabolically and consumed glucose highly, and produced some proteins with molecular weights of 8-89 kDa. Further study needs to characterize these proteins and also to investigate the role of these proteins on MET induction or progression. We also evaluated the possible role of oxidative stress as a tumor favored condition in our observed phenomenon by measuring the total antioxidant capacity in the long cell culture supernatant. Surprisingly, the supernatant exhibited an increased level of antioxidant activity. Giannoni et al. showed the promoting role of oxidative stress on EMT (16). If we consider the MET as a reverse phenomenon of EMT process (17), it can be concluded that redox dominant condition is a facilitating factor for MET process. Further study is required to examine this hypothesis.

Taken together, the data generated by this study showed for the first time that *in vitro* spontaneous MET-like phenomenon occurrence is possible. This phenomenon may be mediated by some small and intermediate size unknown proteins in a prevailed redox condition.

Conflict of interest

The authors declared no conflict of interest.

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