Stable cell line of postnatal dermal fibroblasts as a new experimental model for biomedical skin research

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Introduction

The ability to restore tissue after damage is vital to any organism. Deviations from the normal healing process leads to formation of pathological scars and contractures. Despite the advances in modern medicine, scar complications remain one of the most complex and discussed problems in biomedicine.

Fibroblasts are the main resident cells of connective tissues, including the dermis. Excessive contractile activity of fibroblasts in healing skin wounds and burns is associated with pathological scars. Traditionally, primary cells or a mouse embryonic fibroblast cell line 3T3/NIH have been used for experimental studies of dermal pathologies *in vitro*. Unfortunately, both models have significant drawbacks. However, the use of primary cells is limited by their low passaging ability, while 3T3/NIH cell line does not fully recapitulate the phenotype of dermal fibroblasts. It is also known that before a certain stage of embryonic development the fetus does not form scars, therefore, embryonic fibroblasts should significantly differ from postnatal fibroblasts (Moulin et al., 2001). Thus, a new cell line effectively combining the properties of both primary and immortalized cell lines is highly needed.

Methods

Isolation and cultivation of mouse dermal fibroblasts: Dermal fibroblasts were isolated from C57BL/6 mice (P5) as described earlier (Takashima A., 2001). Mouse dermal fibroblast cells (MDF) were immortalized according to the 3T3-protocol: 3-day transfer, inoculum 3×10^5 cells (Todaro, Green, 1963).

Gel contraction assay: 3T3/NIH, MDF and dp3T3 (dermal postnatal 3T3 fibroblasts, immortalized cell line) cells were seeded within type I collagen gel (0.1 mg/ml). Collagen gel preparation was carried out as described earlier (Bell E. et al., 1979).

MTT-test: The MTT-test was carried out according to the manufacturer's protocol (Sigma). 3T3/NIH, MDF and dp3T3 cells were plated in a 48-well plate with 10,000 cells per well.

Real-time PCR: RNA was isolated using a reagent ExtractRNA (Eurogen). MDF, 3T3/NIH and dp3T3 cells were seeded in a 6-well plate and lysed upon reaching the monolayer. cDNA synthesis was performed using the "MMLV RT kit" (Eurogen) according to the manufacturer's protocol.

Immunocytochemistry: Fibroblasts were seeded on coverslips of 50,000 cells, cultured for 2 days and fixed using a 4% paraformaldehyde solution in phosphate buffered saline (PBS) for 10 minutes. Antibody staining and phalloidin staining were performed according to the Abcam protocol (Immunocytochemistry (ICC) and immunofluorescence (IF) protocol). The nucleus were dyed by Dapi for 1 minute. After dyeing, the cells were flooded with Immumount (Thermo Scientific) mounting medium and viewed on an Axio Imager 2 Research Microscope microscope.

Karyotyping: Preparations of metaphase chromosomes were obtained and chromosome counting was performed on 30 metaphase plates.

Western blot: MDF, 3T3/NIH and dp3T3 cells were plated on 10 cm² Petri dishes and lysed upon reaching the monolayer. After electrophoresis, the proteins were transferred to PVDF membranes.

Results I: Obtaining and characterization of dp3T3 fibroblast cell line

Fig. 1. Obtaining and characterization of the dp3T3 fibroblast line. **A.** dp3T3, MDF and 3T3/NIH fibroblasts morphology comparison after two days in culture. PhC - phase contrast, 10x, 20x - magnification. Scale bar 100 μ m. **B.** Results of the MTT test for dermal fibroblasts at different passages and the 3T3/NIH line, the mean and standard deviation is shown. **C.** Chromosome number. Mediana, the mean and standard deviation is shown.

Results II: Comparison of contractility, α SMA and collagen I expression in 3T3/NIH, MDF and dp3T3 cell lines

Fig. 2. α SMA and collagen I expression analysis in dp3T3, MDF and 3T3/NIH. **A.** Fibroblasts after two days in culture, immunocytochemical staining with α SMA antibodies, 20x, 40x - magnification, scale bar 100 μ m. **B.** Comparison of the contracting ability of dp3T3, MDF and 3T3/NIH fibroblasts, the graph shows the collagen gel area and the standard deviation. **C.** Western blot data for α SMA, standardization on β -actin. **D.** Real-time PCR data for α SMA, standardization for β -actin, the difference is statistically significant at $p < 0.05$. **E.** Real-time PCR data for collagen I, standardization for β -actin, showed no statistically significant difference at $p < 0.05$. **F.** Immunocytochemical staining with collagen I antibodies, 20x, 40x - magnification, scale bar 100 μ m.

Results III: Expressional analysis of fibronectin and profibrotic cytokines (LOX and CTGF) in 3T3/NIH, MDF and dp3T3 cell lines

Fig. 3. Comparative analysis of FN, LOX, CTGF expression, which play an important role in wound healing. **A.** Results of dp3T3, 3T3/NIH and MDF immunocytochemical staining fibroblasts with FN, LOX, CTGF antibodies after two days in culture. 40x - magnification, scale bar 100 μ m. **B.** Real-time PCR data, standardization for β -actin, the differences are statistically significant at $p < 0.05$. **C.** Western blot data, standardization for β -actin.

Conclusions

1. The karyotype of newly obtained dp3T3 line (median 52 chromosomes) is closer to the normal karyotype of the mouse (40) than the karyotype of the 3T3/NIH line (mean 75).
2. The dp3T3 fibroblast line has morphological characteristics more similar to the MDF line than the classical 3T3/NIH line including fibrillar actin microarchitecture.
3. In 3T3/NIH fibroblasts, the expression of α SMA is lower than in dp3T3 fibroblasts and in MDF. Immunocytochemistry data confirmed the data of PCR and Western blot. In 3T3/NIH fibroblasts, the α SMA protein is diffusely located in the cytoplasm of cells, and in dp3T3 and in MDF it is found in stress-fibrils. The dp3T3 line is similar in its contractility to MDF: both have a higher contractile ability than the 3T3/NIH embryonic fibroblast line.
4. The 3T3/NIH, MDF and dp3T3 fibroblast lines are close in expression level of type I collagen, but the dependence of the amount of collagen protein on the culture density is observed only in our dp3T3 fibroblast and MDF line.
5. According to our data, levels of profibrotic cytokines LOX and CTGF, the dp3T3 line is much more similar to mouse dermal fibroblasts than the 3T3/NIH line.

To summarize, dp3T3 is more similar to MDF than to 3T3/NIH that indicate the preservation of tissue-specific characteristics.

The work on creating a new cell line is a complex and multicomponent task, requiring a large number of tests. We successfully carried out the first stage of this work - obtaining a line of immortalized dermal postnatal fibroblasts. The next stage will focus on the evaluation of its tumorigenic potential, the migration properties and overall suitability of this new cell line for skin research and bioengineering.

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