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Controlled formaldehyde fixation of fibronectin layers for expansion of mesenchymal stem cells

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ABSTRACT

Extracellular cell matrices deposited by cells stimulate cell proliferation. However, their generation is cumbersome and time consuming. We show here that controlled fixation of fibronectin layers after coating culture vessels significantly enhances expansion of murine and human mesenchymal stem cells (MSCs) and, to a lesser extent, primary fibroblasts. In contrast, fibronection fixation did not stimulate proliferation of established cancer cell lines. Fixed vitronectin or collagen IV layers also enhanced proliferation of murine MSCs. Thus, controlled formaldehyde fixation of layers formed by fibronectin or some other extracellular matrix components represents a simple and reproducible way to enhance proliferation of primary cells.

Multiple studies demonstrated that the components of extracellular matrix (ECM) produced by cells provide more natural environment than tissue culture-treated plastic for cells grown in vitro [1]. Cell proliferation, in addition to soluble and transmembrane factors, is controlled by ECM. Decellularized matrices obtained after removal of cultured cells support higher proliferation rate of mesenchymal stem/stromal cells (MSCs) [2,3] and some other cell types [4,5]. Fibronectin is among the most important and best studied ECM proteins and exists as a dimer with a number of motifs for binding integrins [6] and other ECM proteins [7]. In addition, fibronectin accumulates multiple growth factors and may enhance their growth-promoting function [8].

Fibronectin is often used for coating culture vessels, and provides increased proliferation for MSCs [9] and other cells [10] but the effects on proliferation are rather weak. It has been shown that ECM layers deposited by cultured cells and prepared using standard decellularization methods can be easily delaminated and lost [11]. Similarly, a rather weak activation of cell proliferation by immobilized fibronectin may be due to losses as a result of non-covalent binding to hydrophilic culture vessel surfaces.

We thus reasoned that a simple crosslinking of immobilized fibronectin layers may increase their retention resulting in higher indexes of cell proliferation. On the other hand, excessive formaldehyde fixation may result in substantial fibronectin modification leading to the loss of activity. To determine the optimal concentrations of formaldehyde during fixation, we seeded MSCs from the adipose tissue of C57Bl/6 mice on tissue culture dishes which were coated with fibronectin (50 μg/ml) and treated with various concentrations of formaldehyde. After one day of culturing under hypoxic (5% O_2) conditions, cell expansion rate was estimated by comparing the seeded and recovered cell numbers (Fig. 1A). The results indicate that unfixed fibronectin layers increase amount of cells produced after 1 day by about 12% compared to control uncoated dishes. Following formaldehyde fixation, cell expansion is significantly stimulated, with optimal fixation conditions (0.2%–0.4%) resulting in increases of about 52–54% compared to control uncoated dishes, and about 35–37% compared to fibronection-coated unfixed dishes.

MSCs, in particular murine ones, are sensitive to oxidative stress and respond to it by reduced proliferation, generation of reactive oxygen species and premature senescence [12]. Thus, murine MSCs and, increasingly, human MSCs are grown under hypoxia conditions. Recently, Fan et al. have shown that embryonic fibroblasts-produced extracellular matrices are able to protect cultured MSC

Abbreviations: ECM, extracellular matrix; MSCs, mesenchymal stem cells; FBS, Fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; DMLG, DMEM-low glucose; DMHG, DMEM-high glucose; TCP, Tissue culture plastic.

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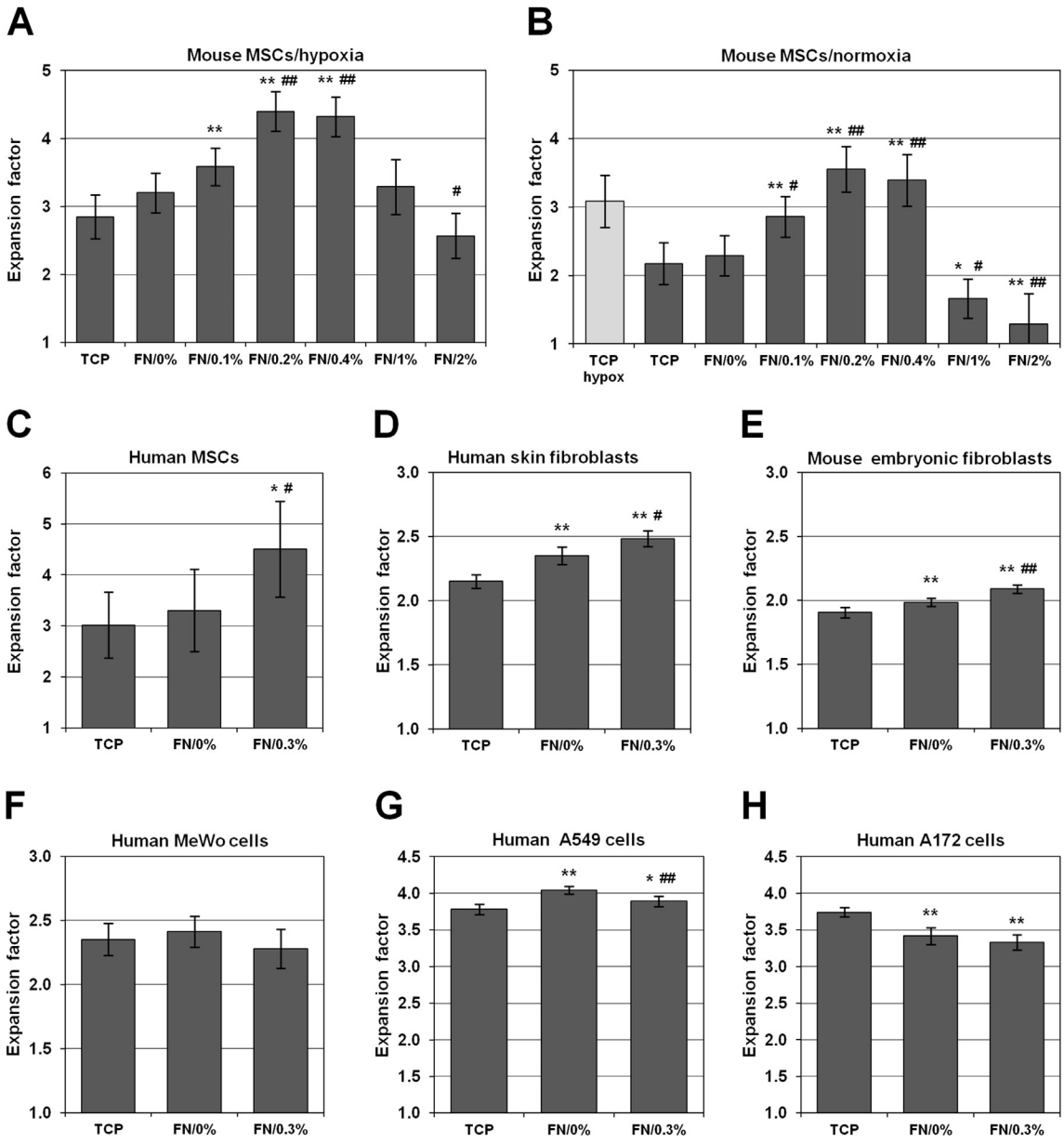

Fig. 1. Controlled fibronectin fixation significantly enhances expansion of primary cells but not of established cell lines. (A) Mouse MSC expansion under hypoxia conditions on control uncoated (TCP, tissue culture plastic) dishes and fibronectin-coated unfixed (FN/0%) and fixed with a various percentages of formaldehyde (FN/0.1% - FN/2.0%) dishes. Cells were seeded at a density 1760 cells/cm^2 and cultured for 24 h in CO_2 incubator (5% CO_2, 5% O_2). Expansion factor (Y axis) reflects the cell growth rate and is calculated as a ratio of a number of the harvested cells to the seeded ones. (B) Mouse MSC expansion under normoxia (5% CO_2, 21% O_2) conditions on uncoated and fibronectin-coated and fixed dishes. Other experimental conditions were identical to those of Fig. 1A. Control cell samples grown at hypoxia conditions (TCP hypox) were included in this experiment for comparison. (C) Human MSC expansion on control uncoated, fibronectin-coated unfixed and fixed with 0.3% formaldehyde dishes. Cells were seeded at a density 690 cells/cm^2 and cultured for 48 h under normoxia conditions. (D) Human skin fibroblast expansion on control (uncoated) and fibronectin-coated unfixed and fixed with 0.3% formaldehyde dishes. Cells were seeded at a density 2760 cells/cm^2 and cultured for 24 h under normoxia conditions. (E) Murine embryonic fibroblast expansion on control (uncoated) and fibronectin-coated unfixed and fixed with 0.3% formaldehyde dishes. Cells were seeded at a density 4140 cells/cm^2 and cultured for 48 h under normoxia conditions. (F) Human MeWo melanoma cell expansion on control uncoated and fibronectin-coated unfixed and fixed with 0.3% formaldehyde dishes. Cells were seeded at a density 2760 cells/cm^2 and cultured for 24 h in CO_2 incubator under normoxia conditions. (G) Human A549 lung carcinoma cell expansion on control uncoated and fibronectin-coated unfixed and fixed with 0.3% formaldehyde dishes. Cells were seeded at a density 4600 cells/cm^2 and cultured for 24 h in CO_2 incubator under normoxia conditions. (H) Human A172 glioblastoma cell expansion on control uncoated and fibronectin-coated unfixed and fixed with 0.3% formaldehyde dishes. Cells were seeded at a density 4600 cells/cm^2 and cultured for 24 h in CO_2 incubator under normoxia conditions. All experiments were performed in quadruplicates, and resulting data are presented as mean ± SD (indicated by bars). Comparisons between groups were performed using Student's t-test. The probability is less than 0.05 (*) or 0.01 (**) relative to control group, and less than 0.05 ($^{\#}$) or 0.01 ($^{\#\#}$) relative to unfixed fibronectin group.

from oxidative stress [13]. We thus analyzed effects of fibronectin fixation on mouse MSC proliferation under normoxia conditions. The results (Fig. 1B) indicate that the positive effects of fixed fibronectin on MSC proliferation were more pronounced under normoxia than hypoxia conditions. In particular, fibronectin layers fixed with 0.2% formaldehyde resulted in 63% increase in cell expansion as compared to uncoated dishes. Thus, the increase in cell expansion factor provided by optimally fixed fibronection layers over tissue culture plastic was about 16% higher for normoxia as compared to hypoxia. The increase in a number of population