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Analysis of the Factors that Limit the Ability of Feeder Cells to Maintain the Undifferentiated State of Human Embryonic Stem Cells

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Human embryonic stem cell (hESC) culture is routinely performed using inactivated mouse embryonic fibroblasts (MEFs) as a feeder cell layer (FL). Although these cells maintain pluripotency of hESCs, the molecular basis for this is unknown. Objectives of this study were to determine whether timing between MEF inactivation and their use as a FL influenced hESC growth and differentiation, and to begin defining the mechanism(s) involved. hESCs were plated on MEFs prepared 1 (MEF-1), 4 (MEF-4), and 7 (MEF-7) days earlier. hESC colony morphology and Oct3/4 expression levels were evaluated to determine the influence of different FLs. Significant enhancement of hESC growth (self-renewal) was observed on MEF-1 compared with MEF-4 and/or MEF-7. Conditioned media (CM) collected from MEF-1 supported significantly better hESC growth in a FL-free system compared to MEF-7 CM. Effects of MEFs on hESC growth were not caused by differences in cell density or viability, although indications of apoptosis were observed in MEF-7. Scanning electron microscopy demonstrated that MEF-7 were morphologically distinct from MEF-1 and MEF-4. Microarray analysis identified 19 genes related to apoptosis with significantly different levels of expression between MEF-1 and MEF-7. Several differentially expressed RNAs had gene ontology classifications associated with extracellular matrix (ECM) structural constituents and growth factors. Because members of Wnt signaling pathway were identified in the array analysis, we examined the ability of the Wnt1 CM and secreted frizzled-related proteins to affect hESC growth and differentiation. The addition of Wnt1 CM to both MEF-1 and MEF-7 significantly increased the number of undifferentiated colonies, while the addition of Sfrps promoted differentiation. Together, these results suggest that microenvironment, ECM, and soluble factors expressed by MEF-1 are significantly better at maintaining self-renewal and pluripotency of hESCs. Our findings have important implications in the optimization of hESC culture when MEFs are used as FL or CM is used in FL-free culture.

Introduction

Embryonic stem cells (ESCs) are typically derived from the blastocyst inner cell mass and provide a potentially unlimited supply of cells that may be directed to differentiate toward specific lineages. Human ESCs (hESCs) were derived and cultured initially on feeder cell layers (FLs), composed of mitotically inactivated (irradiated) mouse embryonic fibroblasts (MEFs) [1]. Although the use of MEF as FL has become the conventional method of hESC culture, human fetal and adult fibroblasts have also been used successfully for hESC culture [2–5], and autogenic FL systems have also been developed [6].

Fibroblasts secrete multiple growth factors, including basic fibroblast growth factor-2 (FGF-2) [7], transforming growth factor-β2 (TGF-β2) [8], extracellular matrix (ECM) proteins [9], activin, Wnts, and antagonists of bone morphogenetic proteins (BMPs) [10], which may support the proliferation of pluripotent hESCs. Culture media previously conditioned by secreted factors from fibroblasts is used for FL-free culture of hESCs on natural derived matrices such as collagen IV,

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fibronectin, laminin, or Matrigel [11,12]. Feeder-free culture systems without fibroblast conditioned medium (CM) have also been described, where hESCs are grown on Matrigel with medium supplemented with knockout serum replacer (KOSR) and high concentrations of FGF-2 (100 ng/mL) [13], or combinations of FGF-2 and Noggin, an antagonist of BMP signaling [14]. However, the background differentiation of hESC colonies cultured in these conditions is higher than those cultured in CM, suggesting that other factors produced by MEF are also required to maintain hESC self-renewal and proliferation. Recently, Ludwig and collaborators [15] developed a defined serum- and animal-free medium containing human albumin and FGF-2, TGF-β, LiCl, gamma-aminobutyric acid, and pipecolic acid. This medium supports derivation and culture of undifferentiated hESCs on a matrix made of a combination of human collagen, fibronectin, and laminin, with the main obstacle being karyotype instability in some of the newly derived cell lines. Consequently, the optimal system for hESC culture has yet to be established. Although continued investigations focus on developing defined synthetic matrices for hESC culture [16,17], further research is needed to define which growth factors and proteins are required in these defined matrix conditions.

To identify key factors secreted by FLs that support hESC self-renewal, studies have compared FLs before and after irradiation [18], supporting versus nonsupporting FLs [19,20], or FLs from different species [10]. This study focused on the ability of the same FL over time following γ-irradiation to support hESC self-renewal. Fibroblasts are mitotically inactivated by irradiation before being used as a FL to avoid overgrowth during the culture of hESCs. However, irradiation may cause changes in fibroblasts that could alter their protein and growth factor secretion, and ultimately affect the hESCs. We report alterations in morphology, in gene expression, and the ability to support hESCs of irradiated MEFs over time. These studies suggest growth factor and matrix combinations that may be critical in maintaining self-renewal and proliferation versus differentiation of hESCs.

Materials and Methods

Human embryonic stem cell culture

Human embryonic stem cell lines hESBGN-01 (NIH code: BG01; BresaGen, Inc., Athens, GA) and H9 (NIH code: WA09; WiCell Research Institute, Madison, WI) were grown on mitotically inactivated MEFs in gelatin-coated tissue culture dishes. Culture medium consisted of Dulbecco's modified Eagle's medium (DMEM)/F12 (Invitrogen, Carlsbad, CA) supplemented with 20% KOSR (Invitrogen; vol/vol), 0.1 mM β-mercaptoethanol, 1 mM L-glutamine, 1% nonessential amino acids (Invitrogen; vol/vol), and 4 ng/mL human recombinant FGF-2 (Invitrogen). Culture medium was changed daily. Undifferentiated hESC colonies were passaged by mechanical dissection into small clumps (100–150 μm) and distributed among FLs. After 7 days in culture, all growing colonies were counted and classified as undifferentiated or differentiated, and means compared using Student's *t*-test. All experiments were repeated at least three times.

Mouse embryonic fibroblast culture

Mouse embryonic fibroblasts were derived from E13.5 CF1 embryos using standard methods [21] by the hESC Core Laboratory at University of Michigan. The MEFs were expanded to passage 3 or 4, γ-irradiated with 4,000 rads, and frozen. For use as feeders, they were plated at a density of 2×10^4 cells/cm^2 on gelatin-coated tissue culture dishes and used as FL: 1 (MEF-1), 4 (MEF-4), and/or 7 (MEF-7) days after plating. The MEF culture medium was composed of high-glucose DMEM, 10% fetal bovine serum (vol/vol), nonessential amino acids, and 200 mM L-glutamine.

Preparation of conditioned media, extracellular matrices, and Matrigel for feeder-free growth of hESCs

To obtain CM, irradiated MEFs (8×10^6 cells) were seeded on to gelatin-coated culture dishes (150 mm; Corning Incorporated, Corning, NY). Twenty-four hours after plating, MEF culture medium was replaced with hESC culture medium (60 mL), and collected the following day. Then, MEFs were fed with hESC culture medium daily and CM were also collected daily for 7 days. CM was stored at –20°C. Before use in hESC culture, CM was supplemented with an additional 0.1 mM β-mercaptoethanol, 2 mM L-glutamine, and 4 ng/mL FGF-2. Extracellular matrices from MEFs were prepared as described by Abbondanzo and collaborators [22]. Briefly, fibroblast cultures were washed twice with phosphate buffered saline (PBS), and then lysed in buffer containing 0.5% (v/v) of Triton X-100/PBS and 35 μL of ammonium hydroxide solution (NH_4OH) per 100 mL for 5 min at room temperature. Plates were washed three times with PBS before use in cell culture. Matrigel-coated plates were prepared with Matrigel (BD BioSciences, San Jose, CA) diluted 1:20 in ice-cold DMEM/F12 at 4°C overnight or at room temperature for at least 2 h.

Analysis of colony differentiation

Human ESC colonies were classified as undifferentiated or differentiated based on morphological observations of definitive colony borders, cell nucleus:cytoplasm ratios, and the presence of embryoid bodies. In the Wnt studies, cell colonies were counted along two orthogonal lines that bisected the culture dish. In all other experiments, all of the colonies present in the 35-mm dish were counted at 6× magnification.

Addition of Wnt signaling agonist and antagonists to hESC culture

To investigate the role of the Wnt signaling pathway in MEF function, we carried out two sets of experiments. In the first experiment, conditioned hESC medium from a line of mouse ESC transfected with a Wnt1 expression construct was added to MEF-1 and MEF-7, and effects on colony differentiation monitored. In the second series of experiments, since the secreted Wnt antagonists, the frizzled proteins Sfrp1, Sfrp2, and Sfrp4 were significantly increased in MEF-7 compared with MEF-1 in the array analysis, we tested their effects on hESC by adding them directly to MEF feeders. Sfrp1, Sfrp2, and Sfrp4 protein (5 μg/mL; R&D systems) were added singly and in combination with MEF-1 and MEF-7 and an assessment of the extent of colony differentiation carried out after 4 days. In an attempt to "rescue" bad MEFs, anti-Sfrp1 antibody (500 ng/mL) was also added to hESC growing on both MEF-1 and MEF-7. Analysis of the number of differentiated

and undifferentiated colonies was carried out as described above; all analyses were carried out in triplicate, a total of 300 colonies were assessed per treatment group.

Oct3/4 expression

Immunocytochemistry (ICC). To detect Oct3/4 expression, hESC colonies were fixed using 2% paraformaldehyde in PBS for 30 min at room temperature. Cells were then permeabilized with 0.1% Triton X-100 for 10 min and blocked with 10% normal donkey serum for 30 min. The Oct3/4 antibody (1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was diluted in 1% normal donkey serum in PBS and hESCs were exposed overnight at 4°C. Primary antibody was detected using the corresponding secondary antibody from Jackson ImmunoResearch (West Grove, PA). Primary antibody was omitted as a negative control. Cell nuclei were stained with Hoechst No. 33258. Samples were imaged using phase-contrast and epifluorescence microscopy.

Western blotting. For SDS-PAGE and western-blot analysis, hESCs were collected by mechanical dissection and placed in lysis buffer composed of 25 mM Tris-HCl (pH 7.4), 20 mM NaCl, 1 mM ethylene diamine tetra-acetic acid (EDTA), 1% Triton X-100, and a cocktail of protease inhibitors (Roche Diagnostics, Mannheim, Germany). Samples were vortexed, placed on ice, sonicated for 10 s; and then denatured at 90°C for 10 min. Equal amounts of protein from each sample were loaded and separated by one-dimensional SDS-PAGE. Resolving gels were cast using 12% acrylamide; stacking gels contained 5% acrylamide. Gels were equilibrated and transferred to Hybond-PVDF transfer membranes (Amersham Life Science, Little Chalfont, Buckinghamshire, England) by Semi-Dry Electrophoretic Transfer Cell (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. Blots were blocked in 5% nonfat milk in Tris buffered saline (TBS) with 0.1% Tween-20 (TBST) at room temperature for 1 h and incubated with Oct3/4 antibody (1:100) diluted in TBST plus 5% nonfat milk overnight at 4°C with rocking. After washing in TBST, blots were incubated with the appropriate horseradish peroxidase–conjugated IgG secondary antibody (1:5,000) at room temperature for 1 h, washed in TBST, and developed with ECL Plus reagents (Amersham Life Sciences) according to the manufacturer's instructions. To verify equal protein loading, blots were stripped for 30 min in a 50°C water bath with agitation in stripping buffer (62.5 mM Tris-HCl [pH 6.7], 100 mM β-mercaptoethanol, and 2% SDS). Stripped blots were blocked in 5% nonfat milk in TBST for 1 h at room temperature, and then incubated with either β-actin antibody (1:250; Sigma, St. Louis, MO) or anti-GAPDH (1:1,000; Sigma) overnight at 4°C with agitation, and processed as described above. Band densities were determined using ImageJ (http://rsb.info.nih.gov/ij) and compared using ANOVA.

Mouse embryonic fibroblast characterization

Cell density and viability. To identify possible differences in cell density and viability between FLs, MEFs were harvested from tissue culture dishes by trypsin–(EDTA; 0.25% trypsin, 1 mM EDTA), washed in PBS, and diluted (1:5) in 0.4% Trypan blue solution. Total cell number and number of cells that incorporated Trypan blue were counted using a hemacytometer. Harvested MEFs were also suspended in PBS containing 1 μg/mL propidium iodide (Sigma) to identify viable cells and subjected to fluorescence-activated cell sorting (FACS) analysis using a COULTER EPICS AltraTM Flow Cytometer (Beckman Coulter). Data were analyzed using EXPO32 multiCOMP software (Beckman Coulter). Student's *t*-test was used for statistical analysis.

Apoptosis assays (cytochrome c analysis)

Mouse embryonic fibroblasts (1×10^7/sample) were harvested and washed in ice-cold PBS. Cells were suspended in 200 μL ice-cold buffer composed of 20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM DTT, 250 mM sucrose, and a cocktail of protease inhibitors. The cell suspension was allowed to sit on ice for 25 min and then cells were disrupted by 10 strokes through a 28.5G needle. The homogenate was centrifuged at 1,000*g* for 10 min at 4°C to remove nuclei. The supernatant was recovered and centrifuged for an additional 30 min at 10,000*g*. The pellet and supernatant were collected as mitochondrial and cytosolic fractions, respectively. To assess apoptosis, SDS-PAGE and western-blot analysis were performed to detect cytochrome *c*. The cytochrome *c* antibody (1:500; BD Bioscience) was generously donated by Dr. Chitra Subramanian and used with an anti-mouse IgG secondary antibody (1:3,000). Blots were stripped and reprobed with β-actin antibody as described above. Band densities were compared using analysis of variance (ANOVA).

Scanning electron microscopy

For scanning electron microscopy, MEFs growing on cover slips were rinsed in serum-free medium and cells fixed for 30 min in 2.5% glutaraldehyde in 0.1 M Sorensen's buffer. After a buffer rinse, MEFs were post-fixed for 1 h in 1% osmium tetroxide in the same buffer. Samples were rinsed in buffer and dehydrated in a graded series of ethanol. After dehydration, cells were treated with hexamethyldisilazane and allowed to air dry. The samples were mounted on SEM mounts, sputter coated with gold-palladium in a Polaron sputter coater, and examined in an Amray 1910 FE scanning electron microscope. Digital images were collected with a Semicaps 2000A Imaging System.

Microarray analysis

Three independent samples of RNAs were isolated from MEF-1, MEF-7, and additionally from three batches of MEFs derived exactly as MEF-1 and MEF-7, but that failed to support undifferentiated growth of hESC regardless of the time in culture as FL (nonsupportive MEF; NS-MEF). RNAs were extracted using the Trizol reagent (Invitrogen, Carlsbad, CA), DNAsed, and gel purified. Three independent samples of RNA (20 μg) from each group of MEFs were labeled, hybridized, and analyzed using the Affymetrix 230 v2.0 mouse arrays by the University of Michigan Microarray Core Facility in the Comprehensive Cancer Center (http://www.michiganmicroarray.com). Statistical analysis was carried out as follows: initially, principal component analyses were carried out on the data to assess clustering. Samples were then analyzed using Pearson's *R* to compare similarities between RNAs. Means from each group were obtained and compared using Student's *t*-test.

Results

Influence of MEFs on growth and undifferentiated state of hESCs

Results from both hESC lines (BG01 and H9) were similar and thus combined for presentation and interpretation. hESC colonies were classified as undifferentiated or differentiated based on the morphological observations of definitive colony borders, cell nucleus:cytoplasm ratios, and the presence of embryoid bodies (Fig. 1). Protein levels of Oct3/4 were also employed to assess the extent of differentiation of the colonies and to validate the microscope observations. When hESCs were cultured on MEF-1, 96.1 ± 1.3% (mean ± SEM) of the colonies maintained an undifferentiated state. In contrast, when hESCs were cultured on MEF-4 or MEF-7, a significant increase ($P \leq 0.05$) in colony differentiation was observed, reducing the percentage of undifferentiated colonies to 74.4 ± 6.2 and 37.9 ± 2.0, respectively. Levels of Oct3/4 protein were significantly ($P \leq 0.05$) reduced by approximately 34% in hESCs cultured on MEF-7 compared with cells on MEF-1 (Fig. 2).

To investigate whether the influence of MEFs on hESCs was due to soluble factors, hESCs were cultured in a feeder-free system (Matrigel) with CM obtained from either MEF-1 or MEF-7. When hESCs were cultured on Matrigel with CM from MEF-1 (CM-1), 78.6 ± 4.3% of the colonies remained undifferentiated, which was significantly greater ($P \leq 0.05$) than the 31.7 ± 5.6% observed in CM from MEF-7 (CM-7). In addition, the expression of Oct3/4 was significantly reduced ($P \leq 0.05$) after 7 days of culture with CM-7 compared with cells cultured in CM-1 (Fig. 3).

A crossover design study using MEFs and CM indicated that the age of feeder MEFs had a strong effect on hESC differentiation. When hESCs were cultured on MEF-1 with either CM-1 or CM-7, 77.2 ± 4.72% and 85.8 ± 1.8% of colonies remained undifferentiated. However, when hESCs were cultured on MEF-7 with CM from either MEF-1 or MEF-7, the percentage of undifferentiated colonies decreased to 22.1 ± 5.9% and 37.0 ± 12%. Statistical analysis between groups showed significant differences ($P \leq 0.05$) in the ability of MEF-1 and MEF-7 to support undifferentiated hESC growth. The analysis of Oct3/4 protein levels showed a significant decrease in cells cultured on MEF-7 in either CM-1 or CM-7 (Fig. 4). When hESC were cultured on ECM deposited by MEF-1 or MEF-7, Oct3/4 protein levels remained high in cells growing on ECM MEF-1 with either CM-1 or CM-7 (Fig. 5). A significant decrease ($P \leq 0.05$) in Oct3/4 protein levels was observed when cells were cultured on ECM from MEF-7 and either CM-1 or CM-7.

Characterization of MEFs

To identify differences between MEF groups, effects of cell density and viability were analyzed. Although irradiated MEFs were seeded at a concentration of 2×10^5/plate, the total number of MEFs that attached and remained on the plates was 1.1×10^5/plate to 1.4×10^5/plate, with a viability of 72–80%. This was similar for all three groups of FLs. Cytochrome *c* analysis revealed a significant increase ($P \leq 0.05$) in γ-irradiated MEFs compared with nonirradiated MEFs; however, no differences were found among MEF-1, MEF-4, and MEF-7. Scanning electron microscopy revealed changes in cell morphology of MEF-7 compared with MEF-1 and MEF-4. MEF-7 often exhibited vertical stratification and a reduced horizontal surface area, indicating cell shrinkage (Fig. 6).

Microarray analysis of MEFs

Microarray data indicated a very high degree of similarity in gene expression pattern between MEF-7 and NS-MEFs (Pearson's $R = 0.972$, $P \leq 0.001$). As expected, MEF-1 also expressed a set of genes in common with MEF-7/NS-MEFs ($R = 0.48$, $P \leq 0.01$). Forty-seven genes were expressed at ≥2-fold in MEF-7/NS-MEFs compared with MEF-1, while 19 genes

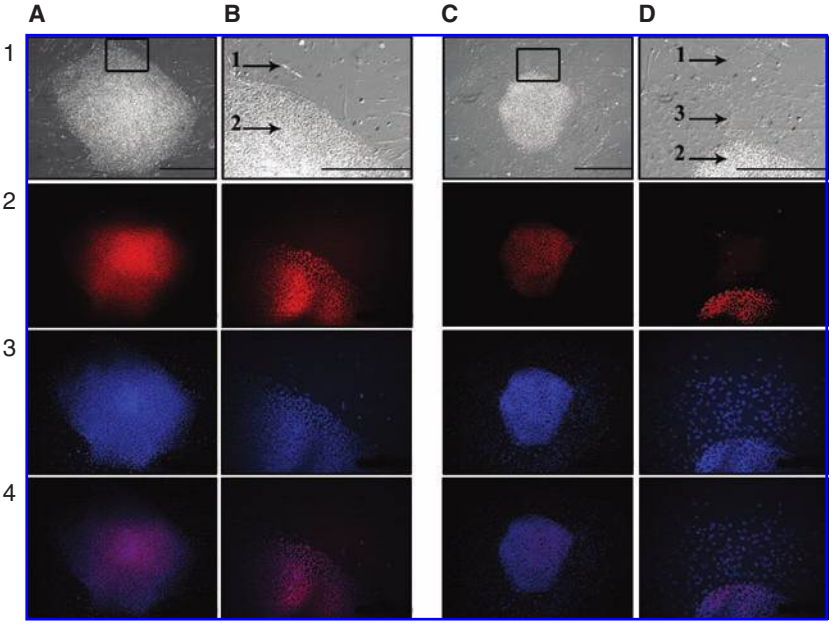

FIG. 1. Human embryonic stem cell (hESC) colony classification. Colonies with clear, defined borders and with all cells in the colony expressing Oct3/4 were classified as undifferentiated (columns **A** and **B**). Colonies with undefined borders with cells showing differentiated morphology at the edges and with some cells lacking Oct3/4 expression were classified as differentiated (columns **C** and **D**). Row 1 shows phase contrast micrographs, row 2 shows ICC localization of Oct3/4, row 3 shows nuclear staining, and row 4 shows merged images from rows 2 and 3. Arrows indicate MEFs (arrow 1), undifferentiated (arrow 2), and differentiated hESCs (arrow 3). Bars: **A** and **C**, 500 μm; **B** and **D**, 200 μm.

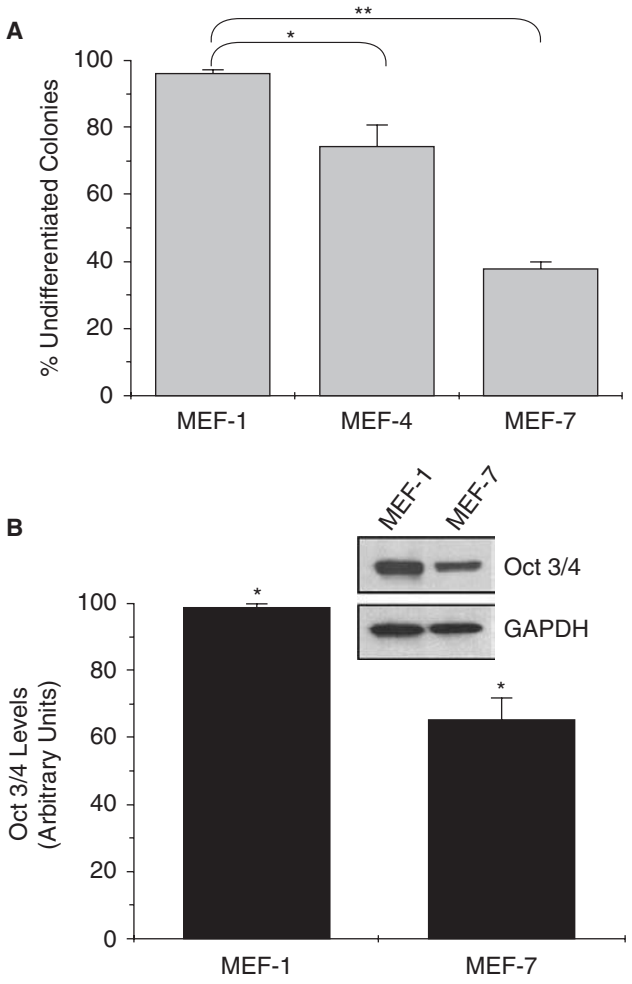

FIG. 2. Effects of mouse embryonic fibroblast (MEF) age post-irradiation on human embryonic stem cell (hESC) growth and expression of Oct3/4. (**A**) Irradiated MEFs were plated 1 (MEF-1), 4 (MEF-4), and 7 (MEF-7) days before use as feeder cell layers for hESC culture. Cells were cultured for 7 days, then colonies classified as undifferentiated or differentiated and means compared between groups using Student's *t*-test. (**B**) There was a significant reduction in Oct3/4 protein levels in hESCs cultured on MEF-7 compared with MEF-1, as quantified by western blot. Data were normalized to GAPDH expression, band intensity from hESCs cultured on MEF-1 was set to 100% and the band intensity from hESCs cultured on MEF-7 was expressed relative to MEF-1. Statistical differences were determined using ANOVA. Inset shows representative Oct3/4 and GAPDH blots. Values are mean ± SEM; * = $P \leq 0.05$; ** = $P \leq 0.005$.

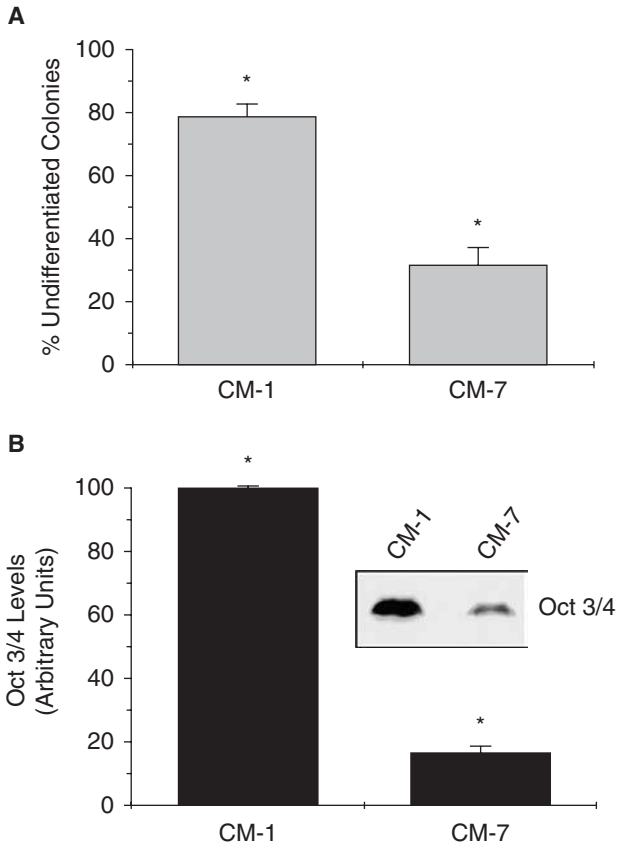

FIG. 3. Effect of mouse embryonic fibroblast conditioned media (MEF-CM) on differentiation of human embryonic stem cells (hESC) grown on Matrigel. (**A**) Conditioned media collected from MEF-1 (CM-1) and MEF-7 (CM-7) were used to culture hESCs on Matrigel. Colonies were cultured for 7 days, then classified as undifferentiated or differentiated and means compared using Student's *t*-test. The growth of undifferentiated hESC colonies was significantly better supported by CM-1 than CM-7. (**B**) There was significant reduction in Oct3/4 protein levels of hESCs cultured in MEF-7 CM compared with cells in MEF-1 CM, as quantified by western blot. Data were normalized to β-actin expression, the band intensity from hESCs cultured in CM-1 was set to 100% and the band intensity from hESCs cultured in CM-7 was expressed relative to CM-1. Statistical differences were determined using ANOVA. Inset shows a representative Oct3/4 blot. Values are mean ± SEM; * = $P \leq 0.05$.

were downregulated ≥2-fold (supplementary table is available online at http://www.liebertpub.com/scd). Among the significantly differentially expressed genes between MEF-1 and MEF-7 were genes with gene ontology identifications of extracellular matrix such as *Col8a2*, *Col11a1*, *Fbn2*, and *Lama4*; growth factors, including *Bmp4*, *Figf*, *Fgf7*, *Fgf9*, and *Gdf10* (*Bmp-3b*); and members of the Wnt signaling pathway and secreted inhibitors of Wnt signaling: *Sfrp1*, *Sfrp2*, *Sfrp4*, and *Fzd4*. Genes involved in apoptosis, necrosis, and DNA damage repair were also upregulated in the MEF-7 group (Table 1).

Role of Wnt signaling in MEF function

Because a number of Wnt pathway members were upregulated in MEF-7 compared with MEF-1, to interrogate the role of Wnt signaling pathways in MEF function, CM collected from mESC-expressing Wnt1 was used to culture hESC on MEF-1 and MEF-7. The addition of Wnt1 CM to cells growing on MEF-1 increased the number of undifferentiated colonies to 97 ± 1.5% compared with 90 ± 1.1% on MEF-1 alone. Similarly, a significant ($P \leq 0.05$) increase in the number of

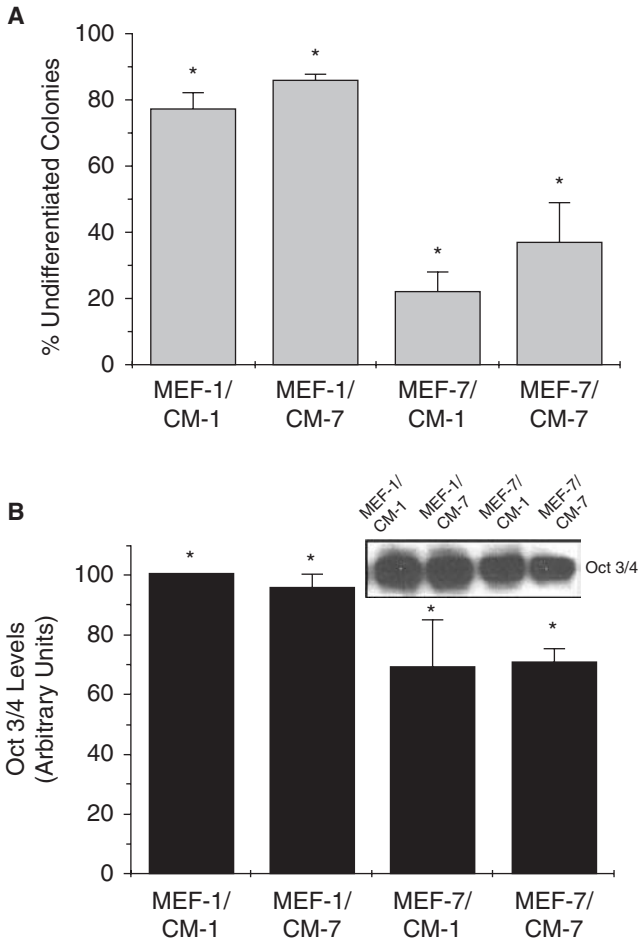

FIG. 4. Effects of conditioned media from MEF-1 (CM-1) or MEF-7 (CM-7) with either MEF-1 or MEF-7 on hESC differentiation. (**A**) Human ESCs cultured on MEF-1 or MEF-7 were grown in CM from MEF-1 or MEF-7 in a crossover design. After 7 days, hESC colonies were classified as undifferentiated or differentiated (mean ± SEM) and compared among groups using Student's *t*-test. The growth of undifferentiated hESC colonies was significantly better supported on MEF-1 than on MEF-7 with either CM-1 or CM-7. (**B**) There was a significant reduction in Oct3/4 protein levels of hESCs cultured on MEF-7/CM-7 compared with other groups, as quantified by western blot. Data were normalized to GAPDH expression and the band intensity from hESCs grown in CM-1 on MEF-1 was set at 100% and the band intensity from hESCs cultured in other conditions was expressed relative to CM-1 on MEF-1. Letters indicate significant differences in expression determined using ANOVA. Inset shows a representative Oct3/4 blot. Values are mean ± SEM; * = $P \leq 0.05$.

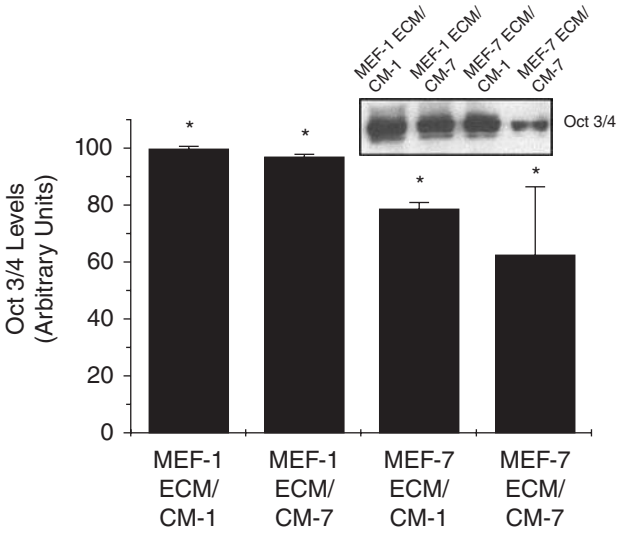

FIG. 5. Expression of Oct3/4 by human embryonic stem cells cultured in conditioned media from MEF-1 (CM-1) or MEF-7 (CM-7) on extracellular matrix (ECM) secreted by MEF-1 or MEF-7. Oct3/4 protein levels were quantified in western blot from hESCs cultured on either ECM MEF-1 or ECM MEF-7 with either CM-1 or CM-7 for 7 days. Data were normalized to GAPDH expression. The band intensity from hESCs cultured in ECM MEF-1/CM-1 was set to 100% and the band intensity from hESCs cultured in other conditions was expressed relative to ECM MEF-1/CM-1. Letters indicate significant differences in expression determined using ANOVA. Inset shows a representative Oct3/4 blot. Values are mean ± SEM; * = $P \leq 0.05$.

undifferentiated colonies was observed when Wnt1 CM was added to cells growing on MEF-7 (83 ± 3.1%) compared with 63 ± 2.3% on MEF-7 alone (Fig. 7). On the other hand, the addition of Sfrp1, Sfrp2, Sfrp4 singly or in combination with MEF-1 reduced the percentage of undifferentiated colonies from 90 ± 1.1% (control) to 79 ± 1.8% (Sfrp1 alone), 80 ± 1.1% (Sfrp2 alone), 72 ± 2.8% (Sfrp4), and 80 ± 2.45% (Sfrp1, Sfrp2, Sfrp4). Furthermore, the addition of anti-Sfrp1 antibody to hESCs growing on MEF-1 and MEF-7 was unable to override the effects of the Sfrps, and also significantly ($P \leq 0.05$) reduced the percentage of undifferentiated colonies to 74 ± 1.3% and 30 ± 2.1%, respectively.

Discussion

A better understanding of the factors by which MEFs, when used as a FL or to obtain CM, support pluripotency of hESC will result in both improved hESC culture methods and understanding of the basic cell biology of hESCs. In this study, the ability of irradiated MEF at different ages to support undifferentiated hESC was evaluated by morphological observations of colonies and using Oct3/4 expression, a marker of ESC pluripotency. Not surprisingly, morphological observations of colonies and visual quantification of colony differentiation were more sensitive measures than the evaluation of Oct3/4 expression, although the trend was similar. More than 90% of the hESC colonies were maintained in an undifferentiated state when cultured on MEF-1, while only ~40% remained undifferentiated when cultured on MEF-7. When hESCs were cultured on Matrigel with CM collected from MEF-7, a high degree of colony differentiation was also observed. When hESCs were cultured on MEF-1 or on ECM deposited by MEF-1 with CM from either MEF-1 or MEF-7, colonies exhibited a lower degree of differentiation. In contrast, regardless of which CM was used for hESC culture on

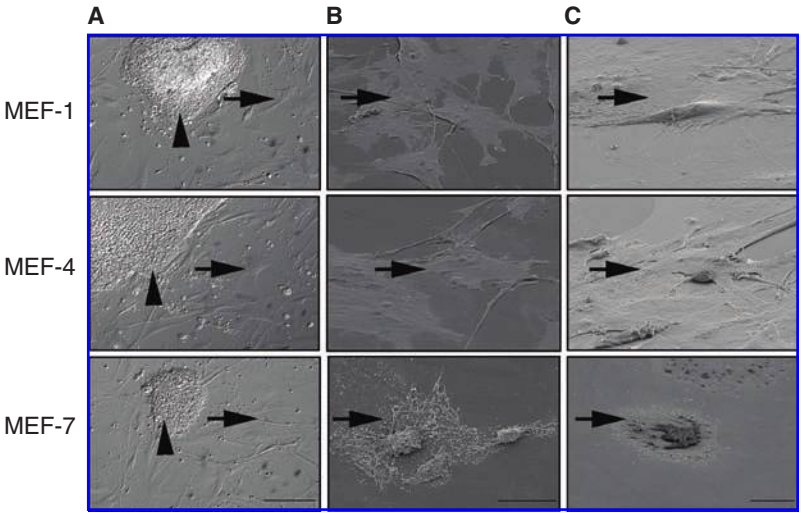

FIG. 6. Morphological changes in post-irradiated mouse embryonic fibroblasts (MEFs) over time. Morphology of MEFs visualized using phase contrast (column **A**) and scanning electron microscopy (columns **B** and **C**) showed significant alterations in cell morphology in MEF-7 compared with MEF-1 and MEF-4. Arrowheads indicate hESC colonies and arrows show fibroblasts. MEF-7 exhibited vertical stratification and a reduced horizontal surface area. Scale bars: (**A**) 200 μm, (**B**) 50 μm, and (**C**) 20 μm.

MEF-7 or on their ECM, significant colony differentiation was observed. These data indicate that the ability of MEFs to support growth of undifferentiated hESCs is compromised over time, and also suggest that soluble factors produced by FLs and likely maintained on their surface override soluble factors present in culture medium. Interestingly, it appears that cell surface bound factors are more critical than secreted factors.

Mitotic inactivation of MEFs by γ-irradiation or using antimetabolites (i.e., mitomycin-C) is necessary for their use as a FL; otherwise, over time replicating MEFs would overgrow the cultures. The exposure of MEFs to irradiation or antimetabolite treatment causes DNA damage by inducing single- or double-strand breaks in DNA and cell cycle arrest [23,24]. In this study, we observed a significant increase in cytosolic cytochrome *c* after irradiation and plating of MEFs, as well as significant changes in cell morphology of MEF-7 compared with MEF-1 and MEF-4. The release of cytochrome *c* into the cytosol and cell shrinkage are indicators of apoptosis [25–28]. Anti-apoptosis genes (*Birc5* and *Hells*) as well as genes related to DNA damage and repair were upregulated in MEF-1, suggesting an immediate response to γ-irradiation of MEF-1 cells. On the other hand, upregulation of several genes involved in apoptosis (*Agtr2*, *Aldh1a1*, *Cd51*, *Casp12*, and *Casp4*) and necrosis (*C1qtnf7*, *Tnfrsf11b*, *Tnfaip6*, and *Fcgr3*) in MEF-7 suggests an increase in programmed cell death. Caspases play an important role in the induction, transduction, and amplification of intracellular apoptotic signals, and both Casp12 and Casp4 in particular have been involved in endoplasmic reticulum stress-induced apoptosis [29–31]. Finally, it has been shown that the binding of tumor necrosis factor to its receptors leads to the activation of caspases, and subsequent apoptosis [32,33].

Several reports that attempt to identify factors produced by FLs that are critical in maintaining hESC self-renewal and pluripotency have demonstrated that fibroblasts secrete FGF-2, TGF-β, activin A, and antagonists of BMP signaling [10,18–20,34]. All these factors have been shown to be involved in hESC self-renewal. For instance, it has been shown that the suppression of BMP signaling and the addition of FGF-2 reduces differentiation of hESCs [14], suggesting that culture in high concentrations of FGF-2 is beneficial for hESC expansion [13]. It has also been demonstrated that FGF-2 cooperates with TGF-β/activin/nodal signaling through Smad2/3 activation to maintain hESC pluripotency [35,36]. Activation of the Wnt signaling pathway is also involved in maintaining the pluripotency of hESCs [37], and it has also been proposed that activin A may regulate FGF-2, Wnt, and BMP pathways in hESCs, maintaining self-renewal and pluripotency [36,38,39]. In this study, the differences in the ability of MEF to support undifferentiated hESC growth prompted us to investigate gene expression patterns of the various MEFs, including fresh batches of MEFs that were derived in an identical way as supportive MEFs, but that did not support the growth of undifferentiated hESCs (nonsupportive: NS-MEFs). Microarray analysis comparing gene expression in MEF-1 versus MEF-7 as well as NS-MEFs identified several differences. The most intriguing observation was the high degree of similarity in gene expression patterns between MEF-7 and NS-MEFs. Among the upregulated genes in MEF-7/NS-MEF population, many were members of growth factor families including IGF, PDGF, and FGF; signaling pathways that have been previously identified in ESC by multiple array–based analyses [40–44]. Among the several genes with growth factor activity that were upregulated in MEF-7 were *Bmp4*, *Figf*, *Fgf7*, *Fgf9*, and *Gdf10* (*Bmp-3b*). With the exception of Bmp4, the role of these growth factors in maintaining pluripotency or promoting differentiation of hESC has not been examined; however, it is known that Fgf7 promotes the proliferation of embryonic pancreatic epithelial cells and prevents the differentiation of these cells into endocrine cells [45,46]. On the other hand, it has been demonstrated that Bmp4 exposure can induce hESCs to differentiate into trophoblast cells [47,48], and Greber and colleagues [49] previously described similar upregulation of Bmp4 in nonsupportive MEF-7. Other genes upregulated on MEF-7/NS-MEF population were members of the Wnt signaling pathway and secreted inhibitors of Wnt signaling, *Sfrps* [50]. This pathway is critically involved in controlling cell proliferation at multiple stages of development, in malignancies and in both mouse and human ESCs. In this study, the addition of Sfrp1, Sfrp2, Sfrp4 singly or in combination significantly increased hESC differentiation while the addition of anti-Sfrp1 to MEF-7

Table 1. Genes Differently Expressed Between MEF-1 and MEF-7 Classified by Gene Ontology

Gene symbol	*Gene ontology classification*	*Unigene*	*Fold change*[a]
Extracellular matrix structural constituent			
Prelp	Proline arginine-rich end leucine-rich repeat	Mm.214514	–1.9
Col8a2	Procollagen, type VIII, alpha 2	Mm.296327	–1.88
Fbn2	Fibrillin 2	Mm.20271	–1.67
Lama4	Laminin, alpha 4	Mm.258065	–1.26
Col11a1	Procollagen, type XI, alpha 1	Mm.209715	–1.27
Col12a1	Procollagen, type XII, alpha 1	Mm.3819	–1.09
Col18a1	Procollagen, type XVIII, alpha 1	Mm.4352	1.05
Col5a3	Procollagen, type V, alpha 3	Mm.334994	1.24
Growth factor activity			
Figf	C-fos–induced growth factor	Mm.297978	–2.21
Igf1	Insulin-like growth factor 1	Mm.268521	–2.13
Nov	Nephroblastoma overexpressed gene	Mm.5167	–2.07
Fgf9	Fibroblast growth factor 9	Mm.8846	–2.01
Bmp4	Bone morphogenetic protein 4	Mm.6813	–1.78
Cxcl1	Chemokine (C-X-C motif) ligand 1	Mm.21013	–1.62
Gdf10	Growth differentiation factor 10	Mm.40323	–1.6
Ogn	Osteoglycin	Mm.4258	–1.49
Angpt1	Angiopoietin 1	Mm.309336	–1.43
Pdgfd	Platelet-derived growth factor, D polypeptide	Mm.141452	–1.42
Fgf7	Fibroblast growth factor 7	Mm.330557	–1.01
Il11	Interleukin 11	Mm.35814	1.22
Ngfb	Nerve growth factor, beta	Mm.1259	1.35
Cntf	Ciliary neurotrophic factor	Mm.290924	1.4
Hbegf	Heparin-binding EGF-like growth factor	Mm.289681	1.52
Apoptosis			
Agtr2	Angiotensin II receptor, type 2	Mm.2679	–5.88
Aldh1a1	Aldehyde dehydrogenase family 1, subfamily A1	Mm.250866	–3.23
Cd5l	CD5 antigen-like	Mm.6676	–2.86
Igf1	Insulin-like growth factor 1	Mm.268521	–2.13
Gas2	Growth arrest specific 2	Mm.207360	–1.93
Angptl4	Angiopoietin-like 4	Mm.196189	–1.73
Il1r1	Interleukin 1 receptor, type I	Mm.896	–1.27
Casp12	Caspase 12	Mm.42163	–1.2
Aplp1	Amyloid beta (A4) precursor-like protein 1	Mm.2381	–1.18
Tnfrsf11b	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	Mm.15383	–1.16
Casp4	Caspase 4, apoptosis-related cysteine peptidase	Mm.1569	–1.15
Ptprv	Protein tyrosine phosphatase, receptor type, V	Mm.4450	–1.14
Fas	Fas (TNF receptor superfamily member)	Mm.1626	–1.04
Prkar2a	Protein kinase, cAMP-dependent regulatory, type II alpha	Mm.253102	1.07
Ngfb	Nerve growth factor, beta	Mm.1259	1.35
Phlda1	Pleckstrin homology-like domain, family A, member 1	Mm.3117	1.4
Hells	Helicase, lymphoid specific	Mm.57223	1.41
Aldh1a3	Aldehyde dehydrogenase family 1, subfamily A3	Mm.140988	1.59
Birc5	Baculoviral IAP repeat-containing 5	Mm.8552	1.62
DNA damage and repair			
Ptprv	Protein tyrosine phosphatase, receptor type, V	Mm.4450	–1.14
Gadd45a	Growth arrest and DNA-damage-inducible 45 alpha	Mm.72235	–1.03
Rad51ap1	RAD51-associated protein 1	Mm.204634	1.05
Ercc1	Excision repair cross-complementing rodent repair deficiency, complementation group 1	Mm.280913	1.1
Exo1	Exonuclease 1	Mm.283046	1.23
Rad51	RAD51 homolog (*Saccharomyces cerevisiae*)	Mm.231	1.32
Uhrf1	Ubiquitin-like, containing PHD and RING finger domains, 1	Mm.42196	1.73

(*Continued*)

Table 1. *Continued*

Gene symbol	*Gene ontology classification*	*Unigene*	*Fold change*[a]
Necrosis			
C1qtnf7	C1q and tumor necrosis factor related protein 7	Mm.275553	–2.41
Tnfrsf11b	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	Mm.15383	–1.16
Tnfaip6	Tumor necrosis factor alpha induced protein 6	Mm.3509	–1.12
Fcgr3	Fc receptor, IgG, low affinity III	Mm.22119	–1.06
Arts1	Type 1 tumor necrosis factor receptor shedding aminopeptidase regulator	Mm.83526	1.02
Tnfrsf22	Tumor necrosis factor receptor superfamily, member 22	Mm.261384	1.2
Tnfrsf23	Tumor necrosis factor receptor superfamily, member 23	Mm.290780	1.25

[a]Log fold change of MEF-1 gene expression compared with MEF-7.

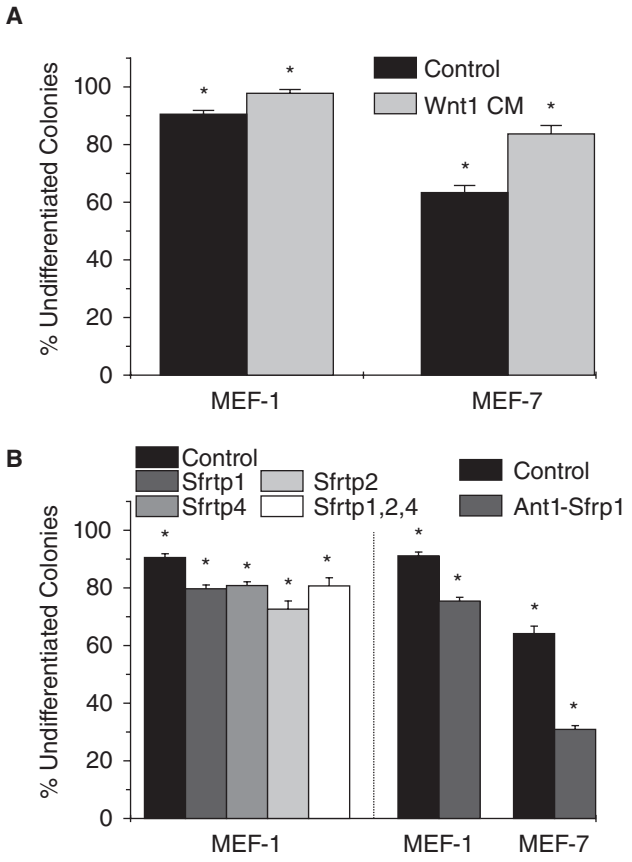

FIG. 7. Effect of Wnt signaling agonists and antagonists on human embryonic stem cells (hESCs) cultured on MEF. (**A**) An increase in the number of undifferentiated colonies were observed when hESCs were grown in conditioned media from mouse embryonic stem cells expressing Wnt1 protein (Wnt1 CM) on either MEF-1 or MEF-7. (**B**) The addition of Sfrp1, Sfrp2, Sfrp4 to MEF-1 promoted hESC differentiation. Similarly, there was a significant increase in colony differentiation was observed when anti-Sfrp1 antibody was added to cultures growing on MEF-1 and MEF-7. Colonies were cultured for 4 days, then classified as undifferentiated or differentiated and compared among groups using Student's *t*-test. Values are mean ± SEM; * = $P \leq 0.05$.

further increased from 36% (MEF-7) to 70% (MEF-7+anti-Sfrpl). This may indicate that some level of Sfrps activity is required to modulate Wnt signaling, or that the response of cells to Sfrp is biphasic [51]. The addition of Wnt1 CM to MEF-7 significantly improved the growth of undifferentiated hESC colonies to a level indistinguishable from culture on MEF-1, and consistently the best substrate for hESC growth was on MEF-1 with Wnt1 CM, supporting growth of 97% undifferentiated colonies. These data suggest that Wnt1 may be an important component of the protein cocktail produced by MEF feeders.

The microarray analysis also identified the upregulation of several extracellular matrix genes in MEF-7, including *Col8a2*, *Col11a1*, *Col12a1*, *Fbn2*, and *Lama4*. A number of inhibitory matrix components were also identified including semaphorin family members and ECM-associated cell death pathway members, particularly clusterin. These data suggest that changes in the ECM production by MEF-7 may play an important role in promoting differentiation. The lack of rescue from differentiation of hESC colonies cultured on MEF-7 or ECM MEF-7 with CM-1 may be explained by the presence of deleterious factors secreted by MEF-7 and the dilution of supportive factors in CM-1. Together, these results suggest that the microenvironment formed by ECM and soluble secreted growth factors from MEF-1 versus MEF-7 differ, which likely explains the reduced ability of MEF-7 to maintain hESC pluripotency. It is also clear from these studies that there is a continuum in these effects, with MEF-1 consistently better, MEF-4 less so, and MEF-7 the least supportive substrate. Somewhat surprisingly, it also appears that factors bound to the cell surface play a more significant role in maintaining pluripotency than secreted factors, but whether aged cells produce toxic factors or fail to produce sustaining factors remains to be determined.

In summary, this study demonstrates that the ability of γ-irradiated MEFs to support hESC growth and pluripotency is compromised over time. Although the viability of MEFs remained high after γ-irradiation, apoptosis is initiated in these cells with cell shrinkage and upregulation of apoptotic genes. These changes in MEFs over time are reflected in the expression of genes that encode growth factors and ECM molecules that may be necessary for self-renewal and proliferation of undifferentiated hESCs. Our findings have

important implications for hESC culture optimization when MEFs are used as FL and when CM is used in FL-free culture. Although undifferentiated hESC colonies cultured on MEF-1 and MEF-7, or with CM-1 and CM-7 have similar morphologies (well-defined borders and high nuclear/cytoplasm ratio), and are able to be selectively passaged to form new undifferentiated colonies that maintain both self-renewal and pluripotency capacity, a significant increase in the number of differentiated cells was observed in cultures with MEF-7 or CM-7. Analysis of factors present in CM from MEF-1 and MEF-7 should identify soluble factors that guide self-renewal of undifferentiated hESCs. In the long term, these data may also aid in the identification of factors that participate in the directed differentiation of hESCs.

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