Int. J. Dev. Biol. 52: 353-363 (2008)
doi: 10.1387/ijdb.082590le

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Comparative study of mouse and human feeder cells for human embryonic stem cells

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ABSTRACT Various types of feeder cells have been adopted for the culture of human embryonic stem cells (hESCs) to improve their attachment and provide them with stemness-supporting factors. However, feeder cells differ in their capacity to support the growth of undifferentiated hESCs. Here, we compared the expression and secretion of four well-established regulators of hESC pluripotency and/or differentiation among five lines of human foreskin fibroblasts and primary mouse embryonic fibroblasts throughout a standard hESC culture procedure. We found that human and mouse feeder cells secreted comparable levels of TGFβ1. However, mouse feeder cells secreted larger quantities of activin A than human feeder cells. Conversely, FGF-2, which was produced by human feeder cells, could not be detected in culture media from mouse feeder cells. The quantity of BMP-4 was at about the level of detectability in media from all feeder cell types, although BMP-4 dimers were present in all feeder cells. Production of TGFβ1, activin A, and FGF-2 varied considerably among the human-derived feeder cell lines. Low- and high-producing human feeder cells as well as mouse feeder cells were evaluated for their ability to support the undifferentiated growth of hESCs. We found that a significantly lower proportion of hESCs maintained on human feeder cell types expressed SSEA3, an undifferentiated cell marker. Moreover, SSEA3 expression and thus the pluripotent hESC compartment could be partially rescued by addition of activin A. Cumulatively, these results suggest that the ability of a feeder layer to promote the undifferentiated growth of hESCs is attributable to its characteristic growth factor production.

KEY WORDS: *human embryonic stem cell, growth factor production, undifferentiated growth*

Introduction

Human embryonic stem cells (hESCs) were originally derived from and maintained in culture systems containing a mouse embryonic fibroblast feeder layer (Thomson *et al.,* 1998). This system has been shown to be efficient for long-term culture of undifferentiated cells, although supportive and non-supportive mouse feeders were also identified (Greber *et al.,* 2007). However, clinical application of hESCs warrants culture of these cells under xeno-free conditions. Therefore, a concentrated effort has been made to develop culture systems that employ feeder cells of human origin. Several different types of human feeder cells have been successfully used for derivation or culture of hESCs. These include fetal muscle and skin cells (Richards *et al.,* 2002), adult skin cells (Richards *et al.,* 2003), foreskin fibroblasts (Amit *et al.,* 2003; Hovatta *et al.,* 2003; Inzunza *et al.,* 2005; Ellerstrom *et al.,* 2006), and adult marrow cells (Cheng *et al.*, 2003). In addition, fibroblast-like cells derived from hESCs have been used as well-defined and supportive autogeneic feeder layers (Xu *et al.,* 2004; Stojkovic *et al.,* 2005; Wang *et al.,* 2005). Adult fallopian tubal epithelial cells, adult muscle cells, adult endometrium-derived cells, and fetal lung cells have also tested, but all were found to be

Abbreviations used in this paper: BMP-4, bone morphogenetic protein 4; ECM, extracellular matrix; FBS, fetal bovine serum; FGF-2, fibroblast growth factor 2; hESCs, human embryonic stem cells; HFFs, human foreskin fibroblasts; KSR, KnockOut serum replacement; SSEA3, stage specific embryonic antigen 3; TGFβ1, transforming growth factor β1.

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Accepted: 15th January 2008. *Published online:* 21 February 2008. *Edited by:* Christine Mummery.

0214-6282/2008/$35.00

Printed in Spain

non-supportive or to only weakly support the undifferentiated growth of hESCs (Richards *et al.,* 2003). The supportive potential of the above-mentioned feeder cell types was indirectly evaluated by measuring expression of stem cell markers on co-cultured hESCs. As a result, the major factors that contribute to suitability of feeder cells for long-term culture of hESCs remain largely unclear.

Ideally, feeder cells play a dual role in maintaining undifferentiated hESCs. They support hESC attachment through expression of adhesion molecules and production of extracellular matrix (ECM). They also support the growth and survival of hESCs through the production of growth factors. In this way, feeder cells create an *in vitro* stem cell niche, which is thought to result from intense interaction between feeder cells and Oct-4 negative

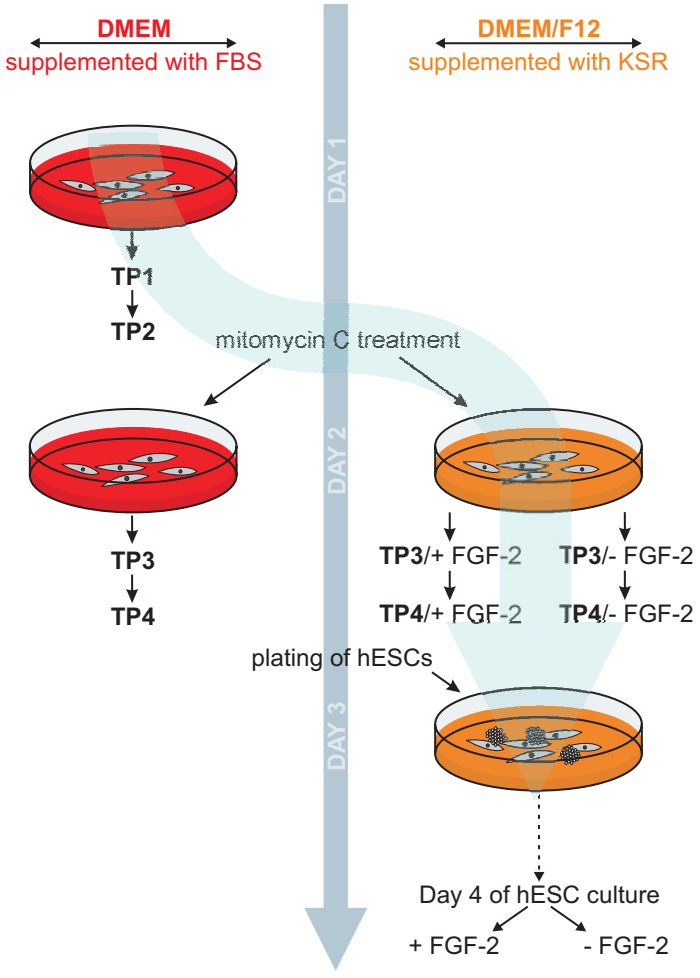


Fig. 1. Schematic of human embryonic stem cell (hESC) culture procedure and media collection time points (TPs). *Conditioned media were collected from fibroblast cultures grown in DMEM with FBS after 6 (TP1) and 24 hours (TP2) of growth. Fibroblasts were then treated with mitomycin C, re-seeded at the same density in DMEM/F12 with KnockOut serum replacement (KSR) (or DMEM with FBS as a control to assess the effect of the media switch on growth factor production), and cultured ± FGF-2. Conditioned media were collected at 6 (TP3) and 24 hours (TP4) of culture. At 24 hours, CCTL 14 hESCs were plated on mouse and human feeder layers, with conditioned medium being collected four days after growth. Transparent arrow indicates the standard protocol used for preparation of feeder layers and plating the hESCs. FBS, fetal bovine serum.*

fibroblast-like cells autologously derived from hESCs (Bendall *et al.,* 2007). Recently, a transcriptome analysis of feeder cells revealed that significant differences exist between supportive and non-supportive human feeder cells. Genes encoding FGF-2, gremlin (a BMP-4 inhibitor), and several ECM components were highly expressed in supportive fetal skin feeder cells (i.e., Detroit 551) compared to non-supportive fetal lung feeder cells (i.e., MRC-5) (Kueh *et al.,* 2006). Consistent with these findings, FGF-2 is known to be involved in self-renewal of hESCs (Dvorak *et al.*, 2005). In this context, exogenous FGF-2 has been identified as a key factor regulating the expression TGFβ1, activin A, and the BMP-4 antagonist, gremlin, in feeder cells (Greber *et al.,* 2007). Nevertheless, the results of this transcriptome analysis are limited, as they do not necessarily reflect concentrations of secreted FGF-2 protein. Proteome analyses of conditioned media from feeder cells revealed that STO mouse embryonic fibroblasts produce insulin-like growth factor binding protein 4 (IGFBP-4) (Lim and Bodnar, 2002) and that human foreskin fibroblasts produce IGFBP-3, IGFBP-6, and gremlin (Prowse *et al.,* 2005). Of potential concern, alterations in synthesis of growth-regulating proteins may occur following mitotic inactivation by mitomycin-C or irradiation. For example, several signaling proteins, including Wnt-3, are newly expressed after irradiation of mouse embryonic fibroblasts (Xie *et al.,* 2004).

Although several studies have investigated feeder-derived signaling molecules that contribute to maintaining the pluripotency of hESCs, many others have concentrated on the regulatory pathways within hESCs that mediate decisions to self-renew or differentiate. For example, activin/nodal and FGF pathways have been shown cooperate to maintain hESC pluripotency (Vallier *et al.,* 2005). Activin A induces the expression of *OCT-4, NANOG, NODAL, WNT-3, FGF-2*, and *FGF-8* in hESCs and suppresses pro-differentiative signals elicited by bone morphogenetic proteins (BMP) (Pera *et al.,* 2005; Xu *et al.,* 2005; Xiao *et al.,* 2006). These regulatory pathways may function in an autonomous intracrine manner, or they may be activated by factors produced by feeder cells or added to the culture medium. The fact that undifferentiated hESCs are difficult to maintain in feeder-free cultures without conditioned media suggests that feeder cells produce most of the signals. The identification of these signals is facilitated by an understanding of the signaling pathways activated in hESCs.

Here, we compared the production of four well-established regulators of hESC pluripotency and/or differentiation among mouse and human feeder cells throughout two hESC passages. We then analyzed feeder layers showing the most significant differences in growth factor production for their ability to support undifferentiated growth of well-characterized hESC line CCTL 14 (Adewumi *et al.*, 2007). Our data revealed key differences in the ability of mouse and human feeder layers to preserve undifferentiated hESCs, differences that may mirror growth factor production of each feeder.

Results

Expression of FGF-2, TGFβ1, activin A and BMP-4 in mouse and human feeder cells

We first determined whether proliferating mouse and human feeder cells included in our panel express *FGF-2*, *TGFβ1*, *AC-*

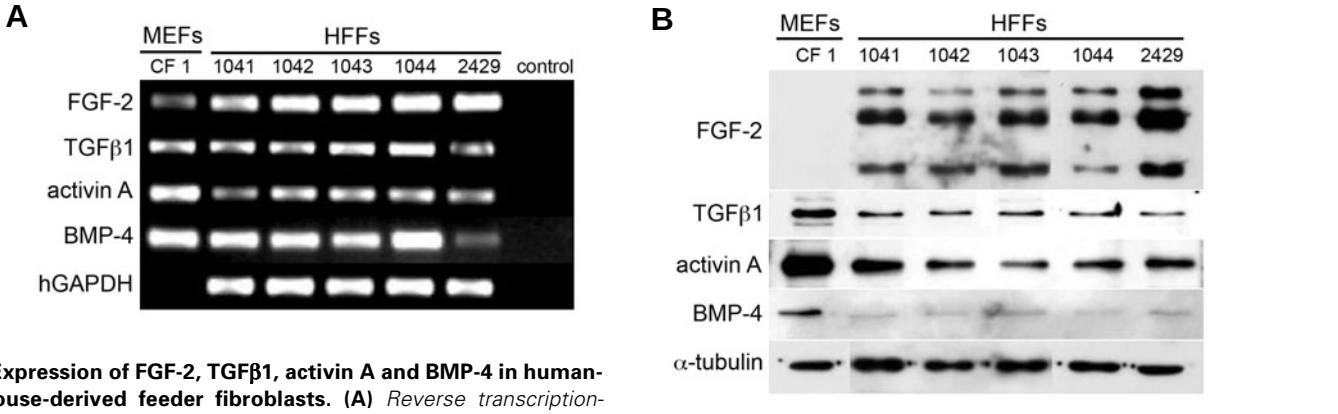


Fig. 2. Expression of FGF-2, TGFβ1, activin A and BMP-4 in human- and mouse-derived feeder fibroblasts. (A) *Reverse transcription-polymerase chain reaction (RT-PCR). Human* GAPDH *served as an internal control, and distilled water served as a negative control.* **(B)** *Western blot analysis. Alpha-tubulin served as an internal control. Note that low and high molecular mass isoforms of FGF-2 (18, 22-22.5, and 24-kDa) are clearly expressed in all lines of human foreskin fobroblasts (HFFs), but are absent in mouse CF 1-derived fibroblasts. Results of RT-PCR and Western blot analyses are representative of two independent experiments. Abbreviations: MEFs, mouse embryonic fibroblasts;* hGAPDH*, human glyceraldehyde-3-phosphate dehydrogenase.*

TIVIN A, or *BMP-4* transcripts. RT-PCR analysis revealed that all five lines of human foreskin-derived fibroblasts used in this study expressed transcripts for all of these growth factors (Fig. 2A). CF 1-derived embryonic fibroblasts also expressed transcripts for these growth factors, including *BMP-4*, which has previously been reported to be absent from mouse feeder cells (Pera *et al.*, 2004). Western blot analysis yielded similar results, with both cell types containing BMP-4 dimers, as well as TGFβ1 and activin A (Fig. 2B). FGF-2 protein was detected only in human feeder cells. This is in accord with previously published findings that FGF-2 protein could not be detected in mouse CF 1-derived feeder cells, although these cells express *FGF-2* mRNA (Dvorak *et al.,* 2005).

Human-derived feeders, but not mouse-derived feeders, produce measurable FGF-2

In accord with western blot results, the CF 1 mouse embryonic fibroblasts did not produce any measurable FGF-2 (Fig. 3). In contrast, six hours after thawing and plating (TP1), all human foreskin fibroblast-derived lines secreted FGF-2 at the levels ranging from 8 to 44 pg/ml (Fig. 3). FGF-2 concentrations then declined, reaching approximately 5 pg/ml at 24 hours in culture (TP2). This decrease was not noticeably altered by inactivation of feeder cells with mitomycin C or by changing fetal bovine serum (FBS)-containing medium to KnockOut serum replacement (KSR)-containing medium. Importantly, neither FBS nor KSR contained measurable levels of FGF-2. A certain amount of FGF-2 was bound to the extracellular matrix synthesized by human feeder cells; however, after 7 days in culture, this amount did not exceed 10 pg per 1 x 10^6 proliferating cells (Supplementary Fig. 1). In the case of both human (except line CRL 2429) and mouse feeders, the FGF-2 concentration increased slightly when hESCs reached higher densities, confirming previous reports that hESCs produce FGF-2 (Dvorak *et al.,* 2005). Notably, and in agreement with previously published data (Levenstein *et al.,* 2006), the addition of FGF-2 to cultures with hESCs, at a commonly used concentration of 4 ng/ml, was associated with rapid degradation or processing of FGF-2, with concentrations being 50 – 60 pg/ml after 24 hours of culture.

Human and mouse feeder cells secrete comparable levels of TGFβ1 that are further increased by exogenous FGF-2

Enzyme-linked immunosorbent assays revealed that TGFβ1 was present at high concentrations in FBS, exceeding 4000 pg/ml. On the other hand, TGFβ1 could not be detected in KSR. The high TGFβ1 concentrations found in media supplemented with 20% of FBS (~800 pg/ml) were augmented by the presence of either mouse or human feeder cells, which produced about 300 and 300 – 800 pg/ml of TGFβ1 every 24 hours, respectively. Mitotic inactivation of feeder cells by mitomycin C suppressed

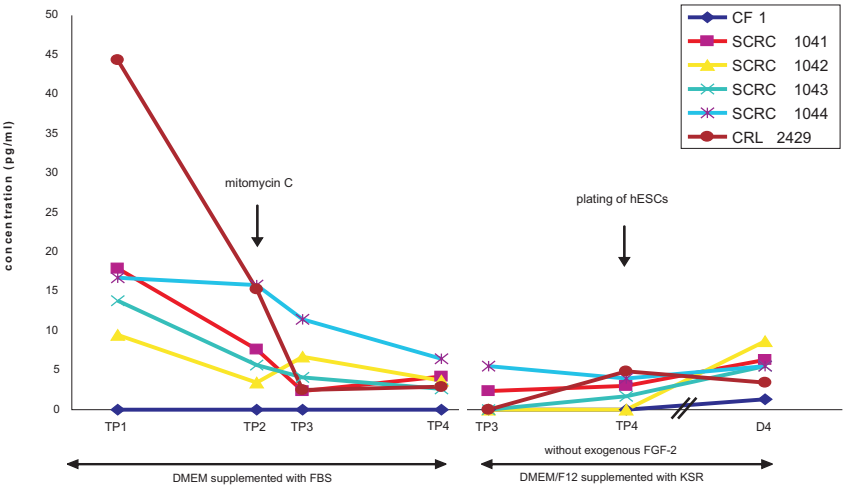


Fig. 3. FGF-2 secretion by human- and mouse-derived feeder fibroblasts. *Left panel shows FGF-2 concentrations, as measured by ELISA, in media from feeder cells cultured in KnockOut DMEM supplemented with FBS. Right panel shows FGF-2 media concentrations from feeder cells cultured in DMEM/F12 supplemented with KSR before and after the plating of hESCs. Similar results were obtained in two independent experiments. Abbreviations: FBS, fetal bovine serum; KSR, KnockOut serum replacement.*

production of TGFβ1 by 5 to 34% (Fig. 4A, left panel, TP2 *vs.* TP4). As expected, exchanging FBS-containing media for KSR-containing media resulted in an additional decline in the TGFβ1 levels. These values were not increased by seeding and subsequent expansion of hESCs in media devoid of exogenous FGF-2 (Fig. 4A, right panel). Based on the previous findings that FGF-2 induces the expression of *TGFβ1* and *ACTIVIN A* genes in feeder cells (Greber *et al.,* 2007), we reasoned that exogenous FGF-2 may also elicit increased secretion of these growth factors into conditioned media. Indeed, KSR- and FGF-2-supplemented media that was conditioned by feeder cells for 24 hours contained 25 – 38% more TGFβ1 than comparable media without FGF-2. A maximum concentration of 400 pg/ml was observed in media conditioned for 24 hours (Fig. 4B). Similar concentrations were found in the dividing cells. Furthermore, a trend towards an increase in TGFβ1 concentrations was also observed in cultures containing FGF-2 and a high density of hESCs (4 days after plating), suggesting production of TGFβ1 by hESCs (Fig. 4B). Interestingly, the human foreskin fibroblast-derived line CRL 2429 exhibited superior production of TGFβ1 at all time points examined, except during hESC co-culture. This unexpected decrease could be explained by either a decrease in viability of mitotically-inactivated CRL 2429 cells after 7 days of culture or an increased utilization/degradation of TGFβ1 by hESCs that are thriving on this feeder layer (see Fig. 6). The latter explanation is supported by the observation that similar decreases in TGFβ1 concentration occur in FGF-2-supplemented, high-density hESC cultures supported by CF 1-derived feeder cells (Fig. 4B), another feeder layer that supports efficient expansion of hESCs (see Fig. 6).

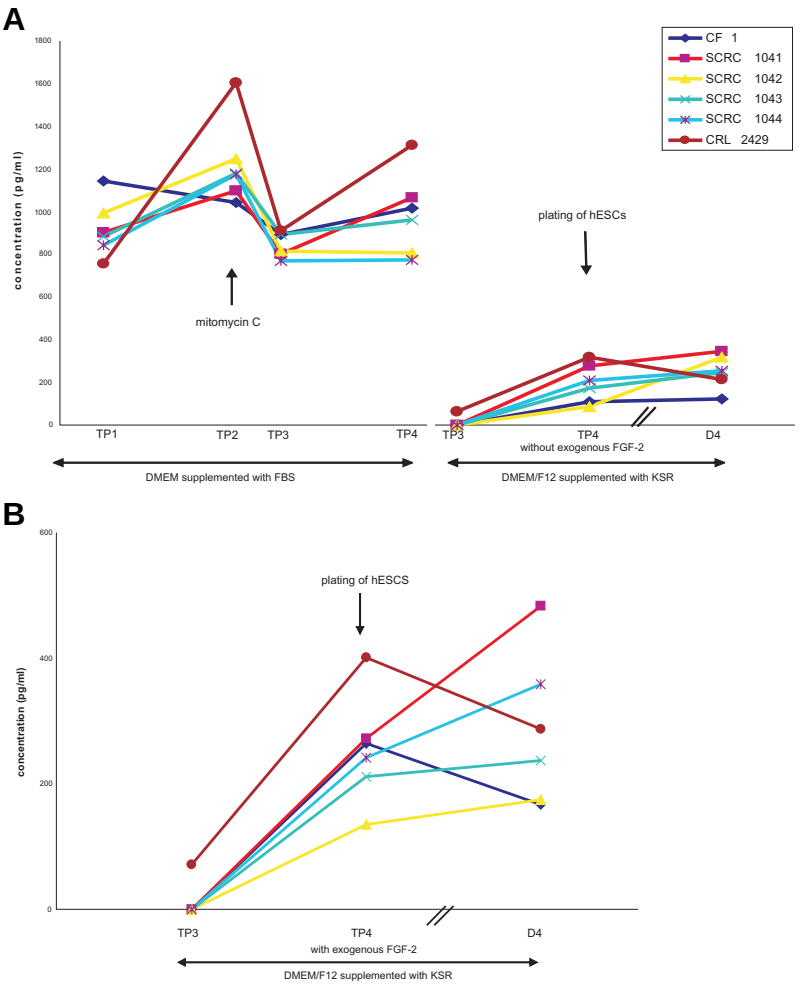


Fig. 4. TGFβ1 secretion by human- and mouse-derived feeder fibroblasts. (A) *Left panel shows TGFβ1 concentrations, as measured by ELISA, in media from feeder cells cultured in KnockOut DMEM supplemented with FBS. Right panel shows TGFβ1 concentrations in cultures grown in DMEM/F12 supplemented with KSR before and after the plating of hESCs. Note the dramatic drop in TGFβ1 concentrations associated with the change in media (800-1300 to 100-300 pg/ml, compare TP4 in the left and right panel).* **(B)** *Media concentrations in cultures grown in DMEM/F12 supplemented with KSR and FGF-2. FGF-2 stimulated the production of TGFβ1 in all feeder layers tested (compare TP4 in A and B). For (A,B), similar results were obtained in two independent experiments. Abbreviations: FBS, fetal bovine serum; KSR, KnockOut serum replacement.*

Production of activin A in mouse-derived feeder is superior to human-derived feeders and is further stimulated by KSR and exogenous FGF-2

Like TGFβ1, activin A was also present in FBS used in this study (780 pg/ml). Activin A concentrations were lower in KSR (390 pg/ml). Accordingly, media with 20% of FBS or 15% KSR was calculated to contain 156 or 58.5 pg/ml activin A, respectively. These values were confirmed experimentally. After 24 hours of culture, FBS-supplemented media conditioned by human feeder cells contained 900—– 3200 pg/ml, while comparable media from mouse fibroblasts contained 4700 pg/ml. Therefore, the net production was 744 – 3044 pg/ml in human feeders and more than 4500 pg/ml in mouse feeder cells. The addition of mitomycin C suppressed the production of activin A in human feeders by about 30 – 70%; however, activin A concentrations remained unchanged by mitomycin C treatment in mouse feeder cells (Fig. 5A, left panel). In human feeder cells, the secretion of activin A was increased from 30 to 100% after the first 24 hours of culture in KSR-containing media. In contrast, exchanging FBS-containing media for KSR-containing media did not alter secretion of activin A in mouse feeder cells (Fig.5A, right panel). Unexpectedly, exogenous FGF-2 did not affect activin A secretion by human feeders, but doubled secretion by mouse feeder cells (Fig. 5B). Finally, in all feeder layers, activin A concentrations were decreased at four days after hESC plating, with decreases being greater in the presence of exogenous FGF-2 (47-60%) than in the absence of FGF-2 (15-33%) (Fig. 5B *vs.* Fig. 5A, right panel). This decrease gradually increased with the number of hESCs and was still apparent at 24 hours, suggesting that activin A was being utilized/degraded by hESCs.

Both human- and mouse-derived feeders secrete extremely low levels of BMP-4

Surprisingly, a highly sensitive ELISA kit designed to recognize active BMP-4 dimers in cell extracts or conditioned media did not detect any measurable level of BMP-4 in FBS or KSR, although BMP-like activity was

previously ascribed to both cell culture supplements (Xu *et al.,* 2005; Kodaira *et al.,* 2006). One possible explanation, at least for FBS, is that BMP-4 exists in serum as part of a large complex with other molecules (Kodaira *et al.,* 2006), which may mask antigenic determinants. However, secreted BMP-4 dimers could not be detected in conditioned media from any of the six types of proliferating feeder cells, despite the presence of BMP-4 mRNA and protein in each (see Fig. 2). Subsequent treatments, including seeding and expansion of hESCs, did not yield any measurable BMP-4 (data not shown). As an alternative approach, heparin-binding fractions were isolated from media conditioned by proliferating high density feeder cells (5 x 10^4 - 3 x 10^5 cells per 1 cm^2) for 4 days, treated with or without reducing agent (2-mercaptoethanol), and analyzed by western blot using anti-BMP-4 antibody that recognizes both the mouse and human protein. As shown in Supplementary Fig. 2, in highly enriched heparin binding fraction of CF 1 feeder cell conditioned media (total protein obtained from 3 ml of conditioned media was loaded per lane) we detected extremely weak band of 36-kDa that corresponds to the migration position of BMP-4 dimer. The 36-kDa bands were scarcely detectable or absent in heparin binding fractions of media conditioned by human feeder cells. The results are consistent with the expression levels of BMP-4 shown in feeder cells (see Fig. 2).

Undifferentiated growth of hESCs on feeder layers that show variability in the production of growth factors

As CRL 2429 is considered a "high producer" and SCRC 1042 a "low producer," both were selected for comparison with mouse CF 1 embryonic fibroblasts. Each feeder type was tested for their ability to support undifferentiated growth of hESCs (line CCTL 14) for at least six passages. As shown in Fig. 6A, morphological differences between hESCs became apparent three days after plating onto CF 1- and human-derived feeder layers. Cells grown on SCRC 1042 fibroblasts formed colonies that were much smaller than those plated onto CRL 2429- and CF 1-derived feeder layers. Nevertheless, the majority of colonies growing on all three types of feeder layers were round and tightly packed (Fig. 6A), suggesting that all remained undifferentiated and that the observed differences were attributable to altered growth rates. This observation was confirmed by cell counts, which showed decreased growth in hESCs maintained on SCRC 1042 feeder cells (Fig. 6B). While simultaneously culturing the same line of hESCs on mouse and human-derived feeder layers, we noticed that those hESCs maintained on human-derived feeders appeared to differentiate more rapidly than hESCs cultured on mouse-derived fibroblasts. To test whether CF 1-derived fibroblasts were more potent in preserving the undifferentiated state of high-density hESC cultures, we assayed the expression of undifferentiated cell markers. The results revealed that SSEA4, TRA-2-54, TRA-1-60, TRA-1-81, and Oct-4 were equally expressed among hESC colonies cultured on all three feeder layers (Fig. 7A and B). On the other hand, SSEA3 was clearly differentially expressed among hESCs maintained on the mouse-derived feeder layers and those maintained on human-derived layers, with SSEA3 expression being scarcely detectable in the latter group (Fig. 7A). Quantification of SSEA3 immunofluorescent labeling by FACS analysis confirmed this result, showing a significantly lower proportion of positive hESCs maintained on either human-derived feeder type that those maintained on mouse feeder cells (Fig. 7C).

The observation that CF 1-derived feeder cells secreted a significantly greater amount of activin A than the human feeder cells (~6.5 ng/ml *vs.*1.5 ng/ml at hESC plating, see Fig. 5B)

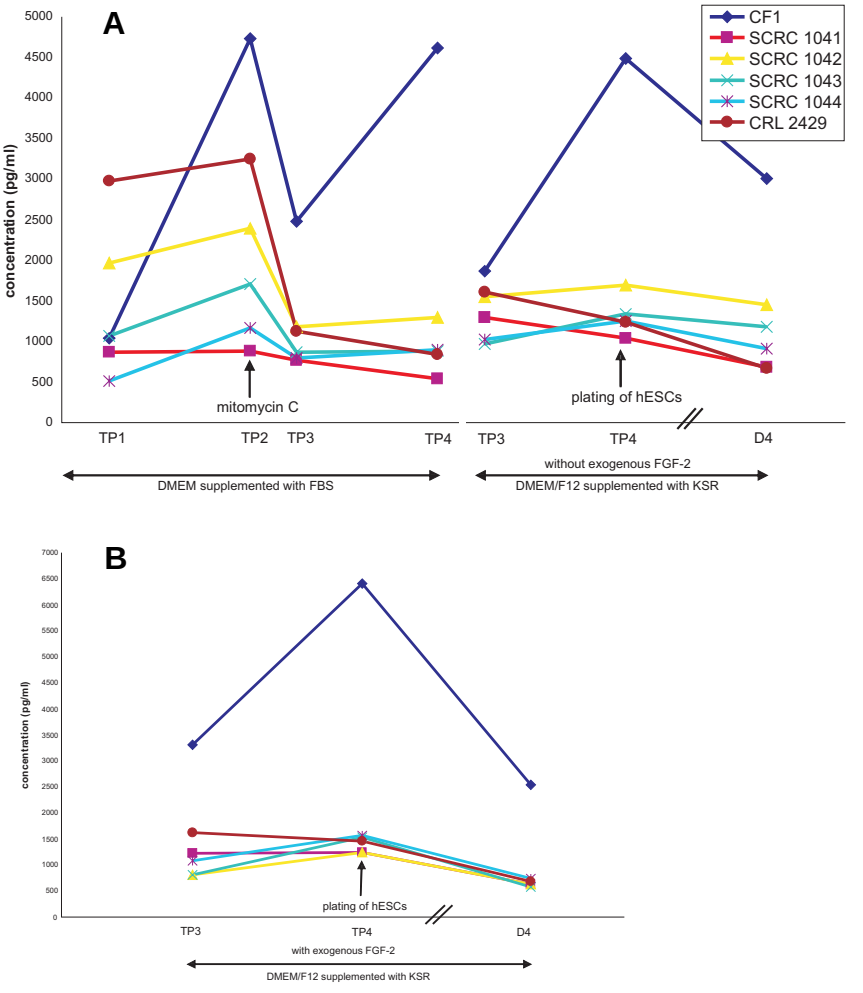


Fig. 5. Activin A secretion by human- and mouse-derived feeder fibroblasts. (A) *Left panel shows activin A concentrations, as measured by ELISA, in media from feeder cells cultured in KnockOut DMEM supplemented with FBS. Right panel shows activin A concentrations in cultures grown in DMEM/F12 supplemented with KSR before and after the plating of hESCs.* **(B)** *Media concentrations in cultures grown in DMEM/F12 supplemented with KSR and FGF-2. Note the superior production of activin A by mouse CF 1-derived feeder cells under all conditions tested. Similar results were obtained in two independent experiments. Abbreviations: FBS, fetal bovine serum; KSR, KnockOut serum replacement.*

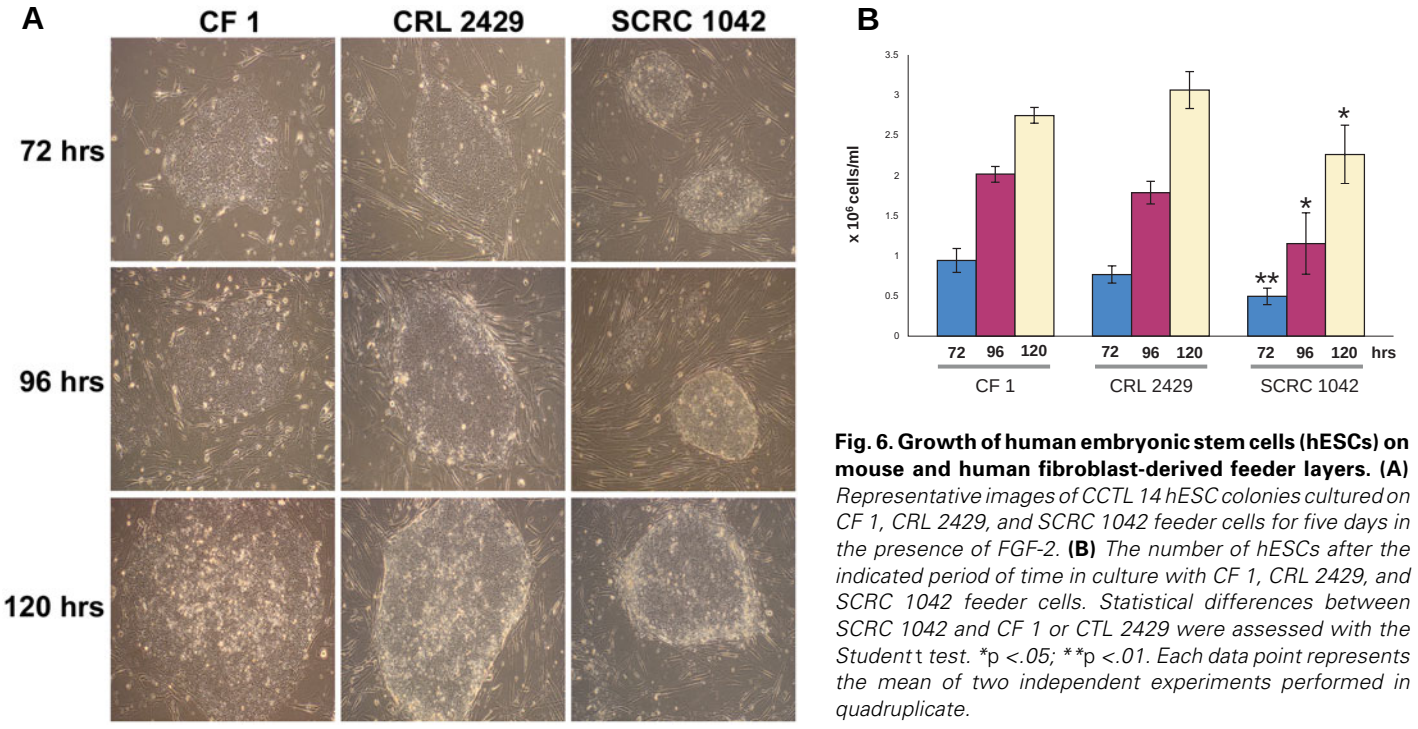


Fig. 6. Growth of human embryonic stem cells (hESCs) on mouse and human fibroblast-derived feeder layers. (A) *Representative images of CCTL 14 hESC colonies cultured on CF 1, CRL 2429, and SCRC 1042 feeder cells for five days in the presence of FGF-2.* **(B)** *The number of hESCs after the indicated period of time in culture with CF 1, CRL 2429, and SCRC 1042 feeder cells. Statistical differences between SCRC 1042 and CF 1 or CTL 2429 were assessed with the Student* t *test. *p <.05; **p <.01. Each data point represents the mean of two independent experiments performed in quadruplicate.*

prompted us to determine whether we could rescue SSEA3 expression among hESCs grown on human-derived feeders by addition of soluble activin A. At concentrations of 5 ng/ml and 25 ng/ml, activin A positively affected the proportion SSEA3-expressing hESCs, although it did not rescue SSEA3 expression to the levels seen in hESCs grown on mouse feeder layers (Fig. 7D). Although this experiment was performed using only one hESC line, the visibility of the effect of activin A is strong enough to justify our concept. Moreover, our quantitative data suggest that, in addition to higher secretion of activin A, mouse feeder cells produce factors, other than those produced by human foreskin-derived feeders, that support undifferentiated growth of hESCs.

Discussion

In this study, we determined the concentrations of soluble FGF-2, TGFβ1, activin A, and BMP-4 in conditioned media from mouse and human fibroblast-derived feeder cells that are widely used for the culture of hESCs. This analysis was performed with conditioned media collected throughout a standard hESC culture procedure. We observed clear differences in growth factor production by both types of feeder cells, as well as a correlation between the production of the well-established regulators of hESC pluripotency and undifferentiated growth of hESCs. These findings could serve as a base for the development and optimization of new feeder cell systems.

Our results revealed two discrepancies in the expression of RNA and protein in feeder cells and conditioned media. First, no FGF-2 protein could be detected in CF 1-derived fibroblasts or in CF 1 fibroblast-conditioned media, although CF 1 fibroblasts expressed *FGF-2* mRNA. This could be explained by the presence of antisense *FGF* mRNAs, which have been implicated in the posttranscriptional regulation of FGF-2 expression (Barclay *et al.,* 2005). These antisense mRNAs could be over-expressed in CF 1-derived fibroblasts (our unpublished data). Second, BMP-4 could not be detected in conditioned media from mouse or human feeders using an ELISA with a sensitivity of 20-30 pg/ml, although both feeder cell types express *BMP-4* mRNA and protein. In this case, the most likely explanation is that both feeder cell types express gremlin (Kueh *et al.,* 2006; Greber *et al.,* 2007), which efficiently blocks BMP-4 secretion by intracellular association (Sun *et al.,* 2006) and thus the concentrations in conditioned media are below the minimum detectable dose. Alternatively, in conditioned media, BMP-4 may be present at high levels, however, in complex with several other molecules, making BMP-4 antigenic determinants inaccessible (Kodaira *et al.,* 2006). However, this second possibility was clearly excluded by western blot analysis of heparin-binding fractions isolated from large volumes of conditioned media.

The most important finding of this study was that mouse and human feeders have very different capacities to produce FGF-2 and activin A. Mouse feeder cells are superior in the production of activin A, but do not secrete any measurable FGF-2. Conversely, human feeder layers have the ability to release up to several tenths pg/ml FGF-2 every 24 hours. Based on our analysis of undifferentiated growth of hESC line CCTL 14 on mouse and selected human feeder layers, we believe that activin A is the most critical factor secreted by feeder cells for the support of hESC undifferentiated growth. We favor this hypothesis since the comparatively higher production of activin A by mouse feeder cells was the only difference that was positively associated with the ability of these cells to promote undifferentiated growth of hESCs. Of course, we cannot exclude that other, as yet identified, factors produced by mouse feeder cells may be more critical. Nonetheless, various combinations of FGF-2, activin A, and TGFβ1 have repeatedly been shown to promote undifferentiated growth of hESCs in feeder-free conditions (Beattie *et al.,* 2005; Amit *et al.,* 2006) and thus, seem to be dominant extrinsic

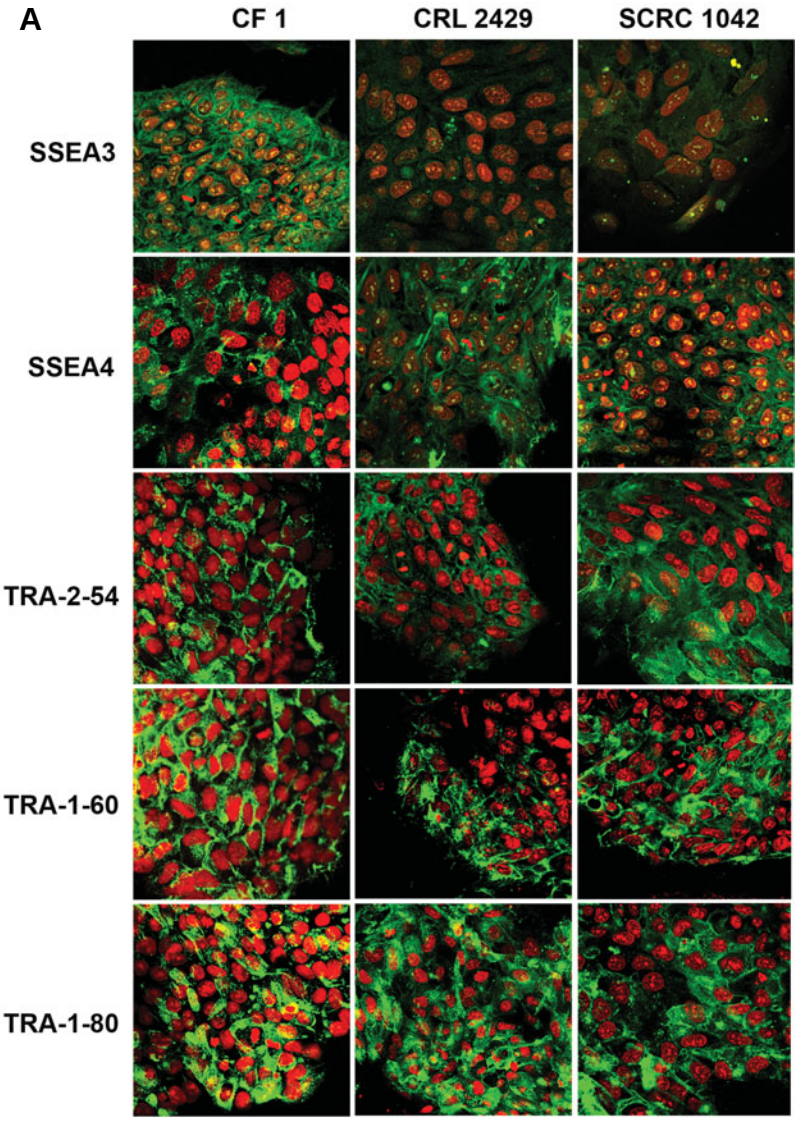
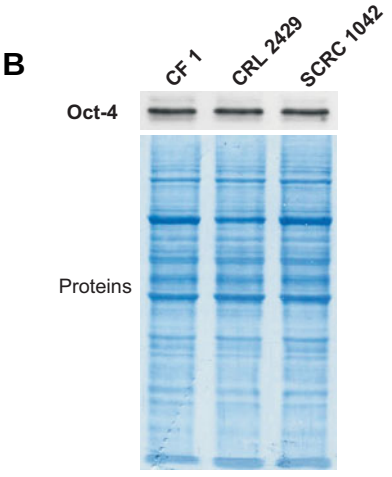


Fig. 7. Expression of markers of undifferentiated human embryonic stem cells (hESCs) in cultures with mouse and human fibroblast-derived feeder layers. (A) *Surface markers (green) were analyzed after 4 days of culture in the presence of FGF-2. Cell nuclei were counterstained with propidium iodide (red).* **(B)** *Western blot analysis of Oct-4 levels in hESCs cultured for five days (upper panel). Equal loading is demonstrated by amidoblack staining of total proteins (lower panel).* **(C)** *Flow cytometric analysis of SSEA 3 labeling on hESCs maintained on feeder cells for several passages (passage number 65-81). Upper panel shows the percentage of positively stained cells at the indicated passage, along with the calculated average. Lower panel shows representative histograms. Green traces represent SSEA 3 and red traces the negative control (IgM isotype control + FITC-conjugated anti-IgM).* **(D)** *Representative flow cytometric histograms showing SSEA3 expression by hESCs grown on human feeder layers in the presence of 5 or 25 ng/ml activin A. The former concentration represents the difference in the concentration of activin A found between mouse and human feeder cells using ELISA. Green traces represent SSEA 3 and red traces the negative control (IgM isotype control + FITC-conjugated anti-IgM). This experiment was repeated three times for each feeder cell line with similar trend (Supplementary Table 2).*



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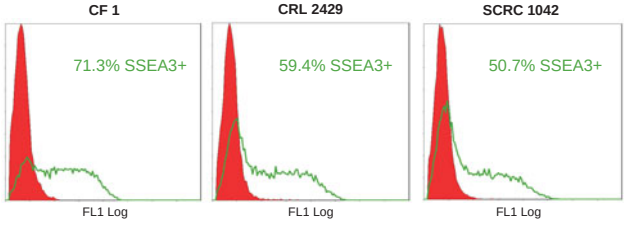
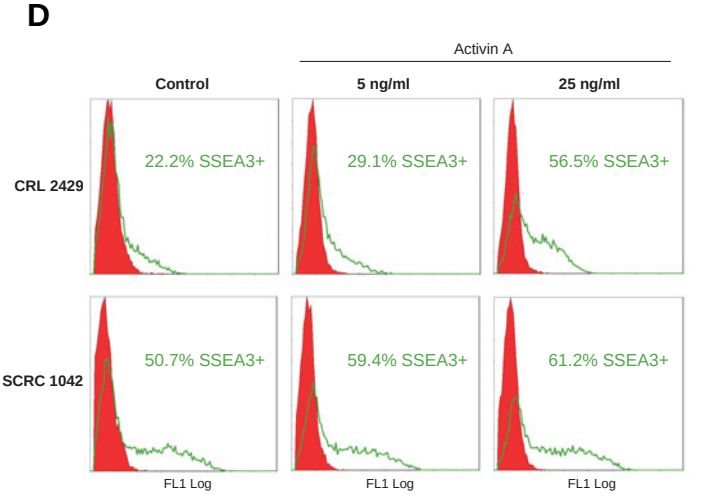
	SSEA3+ (%)						
	Passage number (CCTL14)						
Feeder cells	65	68	69	70	79	81	Average
CF 1	85.4	ND	ND	73.8	71.3	74.9	**76.35**
CRL 2429	36.6	43.9	34.4	51.2	59.4	49	**45.75**
SCRC 1042	35.2	39.5	37.5	30	50.7	55.5	**41.4**





regulators in maintaining hESC pluripotency. It should be noted that hESCs are at least partially autonomous in regulating self-renewal. Otherwise, the dramatic fluctuation in levels of stemness-supporting factors that occurs when exchanging media would be expected to result in more profound changes in hESC phenotypes. Recombinant FGF-2 has been shown to be very unstable in culture media (Levenstein *et al.,* 2006). Here, FGF-2 concentrations that were initially 4 ng/ml decreased to 60 pg/ml after 24 hours, then returned to 4 ng/ml when the medium was changed. Excluding the negligible contribution of FGF-2 that is produced by human feeder cells or that remains bound to the feeder cell ECM, FGF-2 levels fluctuate from concentrations capable of saturating all high-affinity receptors expressed on the surface of hESCs (approximately 1 x 10^4 – 10^6 receptors/cell) (Filion and Popel, 2004; Zehe *et al.,* 2006) to concentrations that are substantially below the capacity of high-affinity FGF-2 binding. Therefore, it would seem that hESCs themselves are not absolutely dependent on exogenous FGF-2, which has been recently suggested to stimulate the secretion of self-renewal promoting factors in feeder cells (Greber *et al.,* 2007) and possibly also in fibroblast-like cells derived from hESCs (Bendall *et al.,* 2007).

Changes in the growth factor concentrations of conditioned media also occur as a result of the treatment of feeder cells with mitomycin C. This is particularly true in human feeder cells, where mitotic inactivation caused a 30 – 70% decrease in activin A. In contrast, mouse-derived feeder cells were resistant to these effects of mitomycin C. Although this decrease in activin A in human feeder cells was partially compensated by the stimulatory effect of KSR, activin A production was not stimulated by the addition of FGF-2 as it was in mouse feeder cells (increase is about 100%), making the difference between mouse and human-derived feeders even more pronounced. In this context, it would be important to analyze the production of growth factors in human and mouse fibroblasts after irradiation, especially since fibroblasts have been shown to express undifferentiated growth-supporting proteins that were absent before irradiation (Xie *et al.,* 2004).

Finally, using hESC line CCTL 14 we have shown that the proportion of cells expressing SSEA3 is significantly smaller in cultures maintained on human-derived feeder layers than in those maintained on mouse feeder layers. This suggests that the efficiency of maintaining undifferentiated hESCs using human foreskin fibroblast-derived feeder layers is not a great as that achieved with mouse feeder cells. In the International Stem Cell Initiative (ISCI) study (Adewumi *et al.,* 2007), detection of SSEA3 reactivity represents the most variable parameter among all the independent hESC lines examined. It was also stated that although globoseries glycolipid antigens SSEA3 and SSEA4 are not essential for pluripotency of hESCs (Brimble *et al.,* 2007), particularly SSEA3 represents a sensitive marker of the most primitive state for hESCs (Enver *et al.,* 2005; Adewumi *et al.,* 2007). Thus, it may be that differences in the expression of SSEA3 between independent hESC lines involved in ISCI study also result from different feeders used in individual laboratories. Here we hypothesize that this reduced ability of human feeders to support growth of SSEA3-expressing pluripotent stem cell compartment results from their decreased expression of activin A. If this is a true, then addition of recombinant activin A should increase the number of SSEA3-expressing cells. Indeed, such as result would have been expected since previous studies have shown that activin A maintains self-renewal and pluripotency of hESCs (James *et al.,* 2005; Vallier *et al.,* 2005; Xiao *et al.,* 2006), even in the absence of feeder layers (Beattie *et al.,* 2005). Theoretically, supplementing cultures with activin A to achieve concentrations present in CF 1 mouse feeder cultures would restore levels of SSEA3 expression to those seen in mouse feeder cultures. The fact that activin A increased the portion of SSEA3-expressing cells, but did not completely rescue it, suggests that mouse feeder cells may secrete other factors important for undifferentiated growth of hESCs. As we realized that many other feeder layer-mediated changes might occur, we recently initiated quantification of *NANOG*, *TDGF*, *POU5F1*, *GABRB3*, *GDF3*, *DNMT3B*, *FGF-4*, *GAL*, and *LEFTB* in hESCs grown on various feeder layers by real-time RT PCR. Possibly one could also employ TaqMan Human Stem Cell Pluripotency Array designed by ISCI for more detailed characterization of undifferentiated state of hESCs.

Together, our results underscore the importance of stem cell niche *in vitro* and provide a foundation for the future study of hESC growth factor signaling in context with specific feeder cell layers. In addition, ELISA-based systems, as used here for the rapid evaluation of growth factor production by feeder cells, will help us to optimize culture conditions.

Materials and Methods

Feeder cells, collection of conditioned media, and culture of hESCs

Mouse embryonic fibroblasts were derived from the CF 1 mouse strain. Pregnant females were sacrificed by cervical dislocation at day 12.5 of gestation, and embryonic fibroblasts were prepared by standard procedures using trypsin-EDTA (Invitrogen, Paisley, U.K., http://www.invitrogen.com). Primary mouse fibroblasts were expanded overnight in KnockOut DMEM supplemented with 20% fetal FBS, L-Glutamine, MEM non-essential amino acids, 1% penicillin-streptomycin (all media components from Invitrogen), and β-2 mercaptoethanol (Sigma-Aldrich, St.Louis, http://www.sigmaaldrich.com) and frozen. For all experiments, first passage mouse fibroblasts were used. Human foreskin fibroblasts (SCRC 1041, SCRC 1042, SCRC 1043, SCRC 1044, and CRL 2429) were obtained from the American Type Culture Collection (Manassas, VA, http://www.atcc.org). Human foreskin fibroblasts were maintained in the same media used for mouse feeder cells and were used at passages 11 – 13.

The time-line for collection of the conditioned media and culture of hESCs is outlined in Fig. 1. Briefly, both types of feeder cells were plated on 6 cm gelatin-coated dishes at a density of 1.4 x 10^4 cells per 1 cm^2. Cell viability and cell counts were determined using ViCELL^{TM} XR (Immunotech a.s., A Beckman Coulter Company, Prague, CR, http://beckmancoulter.com). After 6 and 24 hours, conditioned media were collected (TP1 and TP2, respectively). At 24 hours, fibroblasts were mitotically inactivated by mitomycin C treatment (3 hours, Sigma-Aldrich) and re-seeded at exactly the same density in DMEM/F12 supplemented with 15% KSR, L-Glutamine, MEM non-essential amino acids, 0.5% penicillin-streptomycin, (all media components from Invitrogen), and β-2 mercaptoethanol (Sigma-Aldrich). A subset of these cultures also received 4 ng/ml FGF-2 (Invitrogen). Samples of conditioned media were collected from these cultures 6 and 24 hours later (TP3 and TP4, respectively). At 24 hours, the HESC line CCTL 14 (passage number 60-80) was plated on mouse and human feeder layers at the density of 1.2 x 10^4 cells per 1 cm^2. Culture media were collected from CCTL 14 cultures after 4 days of growth.

Five hundred-microliter aliquots of conditioned media were collected

from each 6 cm dish (containing 5 ml of media), clarified by centrifugation, and stored at -80°C until use. At every collection, dishes were supplemented with media so that the original volume (5 ml) remained unchanged.

Reverse transcription polymerase chain reaction and western blotting

Total RNA was extracted from feeder lysates using the RNeasy Mini Kit (Qiagen, Valencia, CA, http://www1.qiagen.com), and contaminating DNA was digested by DNase I (Qiagen). Equal amounts of RNA (12 ng of RNA per 1μl of total reaction content) were reverse transcribed using M-MLV (Top-Bio, Prague, CR, http://www.top-bio.cz) and oligo-dT (Invitrogen) primers. Amplifications were carried out in a PTC-200 Peltier Thermal Cycler (Bio-Rad, Hercules, CA, http://bio-rad.com) in 50 μl reactions containing 0.5 μM primer and 10 μl diluted cDNA. PCR conditions included a first step of 4 minutes at 94°C, followed by thirty 30-second cycles at 94°C, 30 seconds at 62°C, 1 minute at 72°C, and 5 minutes at 72°C. The PCR products were electrophoresed on 1% agarose gel. Human *GAPDH* served as an internal control. The PCR primers used in this study are shown in online Supplementary Table 1.

For western blot analysis, samples containing equal amounts of total protein were mixed with 2 x Laemmli sample buffer, separated by SDS-PAGE, and electrotransferred onto Hybond P membrane (Amersham Pharmacia Biotech, Buckinghamshire, U.K., http://www1.amershambiosciences.com). Membranes were incubated with one of the following primary antibodies, all of which cross-react with human and mouse proteins: mouse monoclonal antibody to FGF-2 (Sigma-Aldrich, clone FB-8, F6162), biotinylated chicken polyclonal antibody to TGFβ1 (R & D Systems, Minneapolis, MN, http://www.rndsystems.com, BAF240), goat polyclonal antibody to activin A (R & D Systems, AF338), and goat polyclonal antibody to BMP4 (R & D Systems, AF757). Mouse monoclonal antibody to α tubulin (ExBio, Prague, CR, http://www.exbio.cz, clone TU-01, 11-250-M001) was used to normalize loading. Membranes were then incubated with appropriate HRP-conjugated secondary antibodies, and protein bands were visualized using the chemiluminescence detection reagent ECL+Plus (Amersham Pharmacia Biotech).

Enzyme-linked immunosorbent assay

To determine the concentrations of FGF-2, TGFβ1, activin A, and BMP-4 in conditioned media, we used the following commercially available enzyme-linked immunosorbent assay (ELISA) kits: Quantikine HS human FGF basic (HSFB75, sensitivity 0.22 pg/ml); Quantikine human TGFβ1 (DB100B, sensitivity 4.61 pg/ml); Quantikine mouse/rat/porcine/canine TGFβ1 (MB100B, sensitivity 4.61 pg/ml); DuoSet human activin A (DY338, sensitivity 100 pg/ml); and Quantikine human BMP-4 (DBP400, sensitivity given by manufacturer - 1.04 pg/ml; sensitivity under our conditions – 20-30 pg/ml). All kits were purchased from R & D Systems. The Quantikine HS human FGF basic kit was previously determined to accurately measure FGF-2 in samples of mouse origin [15]. We also found that the Quantikine human TGFβ1 and the Quantikine mouse/rat/porcine/canine TGFβ1 kits yielded identical measurements of TGFβ1 in both mouse and human fibroblast-conditioned media; therefore, all subsequent analyses were performed with the Quantikine human TGFβ1 kit. Similarly, the DuoSet human activin A kit measures activin A with high sensitivity also in media conditioned by mouse-derived cells (see Results). As an alternative to the Quantikine human BMP-4 kit, BMP-4 media concentrations were assayed by isolating heparin-binding factors using heparin-coated agarose beads (Sigma-Aldrich) and analyzing the bound fractions for BMP-4 by western blot. All media samples were measured in quadruplicate or greater, and culture experiments were repeated twice with similar results.

Immunocytochemistry and FACS analysis

HESCs growing on either mouse or human feeder layers were fixed with an ice-cold mixture of 95% ethanol and 1% acetic acid, slowly rehydrated, blocked with 5% normal goat serum in PBS (pH 7.4), and incubated with primary antibodies diluted in blocking solution. Primary antibodies included rat monoclonal antibody to SSEA3 (Chemicon International, Temecula, CA, http://www.chemicon.com, MAB4303), mouse monoclonal antibody to SSEA4 (Chemicon, MAB4304), mouse monoclonal antibody to TRA-2-54 (Chemicon, MAB 4354), mouse monoclonal antibody to TRA-1-60 (Chemicon, MAB4360), and mouse monoclonal antibody to TRA-1-81 (Chemicon, MAB4381). Unbound antibody was removed by washing, and cells were incubated with secondary antibodies conjugated to fluorescein isothiocyanate (FITC). Cell nuclei were stained with propidium iodide, and cells were mounted in Mowiol containing 1,4-diazobicyclo-[2.2.2.]-octane to prevent fading. Microscopic analysis was performed using an Olympus FluoView 500 laser scanning microscope (Olympus C&S Ltd., Prague, CR, http://www.olympus.cz). Flow cytometric evaluation of SSEA3 expression among hESCs was performed using a Cytomics FC500 cytometer (Immunotech a.s.). Briefly, cells were incubated with 0.5% Trypsin/EDTA (Invitrogen) for 2 – 3 minutes, and the detached hESC colonies were gently pipetted to form a single-cell suspension. Trypsin activity was then inactivated with FCS, cells were rinsed with wash buffer (5% FCS, 0.1% sodium azide in Ca^{2+}, Mg^{2+}-free PBS), and equal amounts of cells diluted in wash buffer were incubated with primary antibody to SSEA3 (Chemicon). The primary antibody was detected with FITC-conjugated antibody to rat IgM (Immunotech a.s., PN IM1624), and data were analyzed using CXP software (Immunotech a.s.).

Acknowledgments

We thank J. Jaros for help with FACS analysis. This research was supported in part by Ministry of Education, Youth, and Sport of the Czech Republic (MSM0021622430, LC06077, 1M0538), Grant Agency of the Czech Republic (305/05/0434), Academy of Sciences of the Czech Republic (AV0Z50390512, AV0Z50390703), and EC FP6 (contract LSHG-CT-2006-018739) funding.

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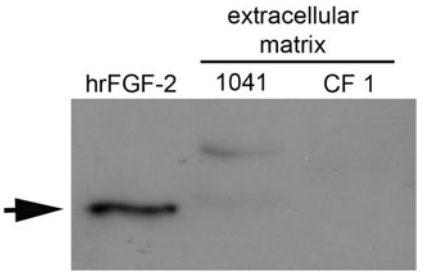
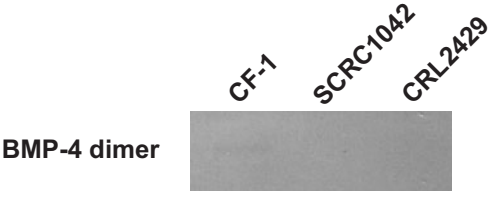
Supplementary Material





Supplementary Fig. 1 (Left). FGF-2 secereted by human feeder cells is partially bound to the extracellular matrix. *To isolate protein from extracellular matrix, fibroblasts were grown for 48-72 hours after reaching confluence, media was removed, and dishes were rinsed twice with ice-cold Ca^{2+}, Mg^{2+} -free PBS, then incubated in Ca^{2+}, Mg^{2+} -free PBS for 30 minutes at 37ºC. Fibroblasts were harvested by gentle pipetting, and the cell-free fraction that remained on dishes was extracted with 2x Laemmli sample buffer and subjected to western blotting for FGF-2. A weak band migrating at 18-kDa was observed in SCRC 1041 human fibroblasts. A 24-kDa band most likely representing FGF-2 complexed with proteoglycans or their fragments was also present. As expected, no bands were detected in mouse CF 1-derived feeder cells. Human recombinant FGF-2 (200 pg per lane) was used as positive control. The amount of FGF-2 that remained bound to the extracellular matrix was estimated by comparing the band densities of a known amount of FGF-2 and a fraction of extracellular matrix obtained from 1 x 10^6 human fibroblasts. Abbreviations: hrFGF-2, human recombinant FGF-2.*

Supplementary Fig. 2 (Right). BMP-4 is scarcely detectable in fraction of heparin-binding factors isolated from media conditioned by mouse and human-derived fibroblasts. *To isolate heparin-binding factors from conditioned media, mouse and human fibroblasts were plated at the density of 5 x 10^4 per cm^2 and grown for 4 days until reaching 3 x 10^5 per cm^2, conditioned media was collected and incubated with heparin-coated agarose beads overnight at 4ºC. The fraction of heparin-binding factors was extracted with 2x Laemmli sample buffer with or without β-2 mercaptoethanol and subjected to western blotting for BMP-4. The sample obtained from 3 ml of conditioned media was loaded per each lane. A weak band migrating at 36-kDa was detected in media from CF 1-derived fibroblasts. Extremely weak signal at about the level of visibility was observed in media conditioned by SCRC 1042 or CRL 2429 human fibroblasts.*

SUPPLEMENTARY TABLE 1

SEQUENCES OF PRIMERS USED FOR REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION

Gene		Sequence (5´ to 3´)	product size (bp) human	mouse
FGF-2	F	GCAGAAGAGAGAGGAGTTGTGTC		
	R	ACTGCCCAGTTCGTTTCAGT	202	202
TGFβ1	F	GTCACCCGCGTGCTAATG		
	R	CAGAAGTTGGCATGGTAGCC	644	644
activin A	F	AGAAGAGACCCGATGTCACC		
	R	ACAGGTCACTGCCTTCCTTG	237	237
BMP-4	F	GACTTCGAGGCGACACTTCT		
	R	CCTGGGATGTTCTCCAGATG	308	311
hGAPDH	F	AGCCACATCGCTCAGACACC		
	R	GTACTCAGCGCCAGCATCG	302	-

Abbreviations: F, forward primer; R, reverse primer; *hGAPDH*, human glyceraldehyde-3-phosphate dehydrogenase.

SUPPLEMENTARY TABLE 2

EXPRESSION OF SSEA3 (%) IN hESCs GROWN ON HUMAN FEEDER LAYERS WITHOUT ACTIVIN A AND IN THE PRESENCE OF 5 OR 25 NG/ML ACTIVIN A

		SSEA3+ (%)		
		Activin A		
Feeder cells	Passage number (CCTL14)	0 ng	5 ng	25 ng
CRL 2429	62	40.6	47.6	50.4
	64	22.2	29.1	56.5
	65	25.6	39.5	45.8
SCRC 1042	62	50.7	59.4	61.2
	64	21	29.9	41.7
	65	26.4	31.4	37.4

Representative histograms as shown in Fig. 7D are marked in red.

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