Int. J. Dev. Biol. 54: 1329-1336 (2010)
doi: 10.1387/ijdb.092854nk

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Role of TGFβ and myofibroblasts in supporting the propagation of human embryonic stem cells *in vitro*

NEERAJ KUMAR, PRASAD PETHE and DEEPA BHARTIYA*

Stem Cell Biology Department, National Institute for Research in Reproductive Health, Parel, Mumbai, India

ABSTRACT The feeder layer constitutes a prerequisite for the undifferentiated proliferation of human embryonic stem (hES) cells *in vitro*. However, a few feeders have been reported to be non-supportive in nature, suggesting that these feeders exhibit a different transcriptome and proteome, in comparison to their supportive counterparts. In an attempt to identify factors required for undifferentiated growth and many downstream applications of hES cells, transcriptomes of supportive (mouse fibroblasts derived from 13.5dpc embryos and human fetal fibroblasts) and non-supportive (mouse fibroblasts derived from 18.5dpc embryos) feeders were analyzed. Furthermore, the parallel correlation of data generated in the microarray study with the published proteome data of supportive feeder fibroblasts, helped us to focus on the proteins which seem to be likely candidates in supporting the undifferentiated expansion of ES cells *in vitro*. Our results indicated that TGFβ and its associated signaling molecules facilitate the undifferentiated proliferation of hES cells *in vitro*. The transient differentiation of feeder fibroblasts into myofibroblasts may be the decisive factor for a feeder layer to be supportive or non-supportive in nature. We propose that the microenvironment of feeder myofibroblasts dictates TGFβ to support proliferation and apparently plays the contradictory role of facilitating differentiation when feeder support is withdrawn, possibly by acting through different signaling mechanisms.

KEY WORDS: *embryonic stem cell, feeder layer, microarray, TGFβ, myofibroblast*

In order to realize full clinical potential of human embryonic stem (hES) cells, a major challenge lies in its large scale production for transplantation which is restricted by the use of feeder layer. Mouse embryonic fibroblasts (MEF), although considered the ideal feeder for supporting hES cell growth, carry a potential risk of transferring animal pathogens to the hES cells and thus making them unsuitable for clinical use. Moreover, release of nonhuman sialic acid Neu5Gc from MEF make hES cells more immunogenic and thus possible rejection at the time of transplantation (Martin *et al.* 2005). Human feeders from various sources have also been used for hES cells derivation and culture e.g. fibroblasts obtained from fetal muscle, skin, lung of which fetal lung fibroblasts were reported to be non- supportive (Richards *et al.* 2003). Thus it becomes pertinent to identify the proteins/ factors that are highly expressed/ produced by feeder fibroblasts that facilitate undifferentiated proliferation, expansion and maintenance of pluripotency of embryonic stem cells *in vitr*o, with the hope to further refine feeder free culture protocols in future.

Studies are available in literature which aimed to identify the secretory factors in the conditioned medium using tools like 2-DE MALDI TOF/ TOF (Lim *et al.* 2002) and 2D LC MS/MS (Prowse *et al.* 2005, 2007). The main problem encountered in these studies was the presence of albumin and other bovine serum proteins that mask the separation of other low abundant and interesting proteins on 2-DE gels. In order to overcome this problem, cells were serum deprived for 16 hrs prior to analysis but this has been invariably associated with the expression of several stress- related proteins (Lim *et al.* 2002). Non- optimal serum deprivation induces stress resulting in cell lysis and several intracellular proteins were identified during proteome analysis e.g. metabolic enzymes, heat shock proteins and nucleus-associated proteins.

Abbreviations used in this paper, α-SMA, alpha smooth muscle actin; BMP, bone morphogenic protein;dpc, days post coitum; ECM, extra cellular matrix; FGF 2, fibroblast growth factor-2; hES, human embryonic stem; HFF, human fetal fibroblasts; MEF, mouse embryonic fibroblasts; SPARC, secretory protein acidic and rich in cysteine; TGFβ, Tumor growth factor beta; GAPDH, Glyceraldehyde 3 –phosphate dehydrogenase; EDTA, Ethylene diamintetra acetic acid; PVDF, Polyvinylidene fluoride; NFDM, Non fat dried milk; TBST, Tris-buffered saline.

***Address correspondence to:** Deepa Bhartiya. Stem Cell Biology Department, National Institute for Research in Reproductive Health, Parel, Mumbai 400 012, India. Fax:. +91-222-412-3492. e-mail: deepa.bhartiya@yahoo.in

Accepted: 22 September 2009. *Final author corrected PDF published online:* 20 July 2010.

ISSN: Online 1696-3547, Print 0214-6282

The presence of extracellular proteases was also suspected to cause lysis of certain membrane bound proteins. However, it is well accepted that proteins belonging to the category of extracellular matrix proteins, cell cytoskeleton and growth factors are most likely involved in the maintenance of hES cells growth since they are associated with variety of events including cell adhesion, cell proliferation, differentiation etc. (Lim *et al.* 2002; Prowse *et al.* 2005, 2007; Kueh *et al.* 2006; Eiselleova *et al.* 2008).

An earlier report from our laboratory has shown that feeder layers derived from 13.5dpc CF-1 mouse embryo and human fetal fibroblasts (HFF) are supportive for hES cells culture (Kumar *et al.* 2009) whereas the feeder layers derived from 18.5dpc mouse embryo exhibit massive differentiation in hES colonies beyond 1-2 passages. Thus in an effort to identify potential candidates having role in self renewal of hES cells, we studied the transcriptome of supportive and non-supportive feeder fibroblasts by microarray analysis and correlated with earlier reported proteomics data of factors detected in the conditioned medium of supportive feeders.

Results

Feeder layers

The hES cell colonies appear round in shape on 13.5dpc MEF feeder whereas on the human feeder layers they assume banana-like appearance (Fig 1 A,C,D,F). They could be easily passaged in an undifferentiated state on both MEF and HFF. When hES cells were cultured on 18.5dpc MEF, colonies underwent massive differentiation within 1- 2 passages (Fig1 B,E).

Microarray analysis

A total of 739 genes were found to be differentially expressed between 13.5dpc and 18.5dpc MEF of which 416 were up-regulated in 13.5dpc MEF and 323 genes in 18.5dpc MEF. Differential expression of genes equal to or greater than 1.5 fold (log 2) was considered biologically significant for functional analysis. Such up-regulated 147 genes and 43 genes in 13.5dpc MEF and 18.5dpc MEF respectively were annotated at high stringency setting (P<0.01) provided in the Biointerpreter tool. The genes were clustered into pathway analysis, functional categories, cellular and chromosomal localization etc. Interestingly none of the cellular pathways were found to be significantly activated in 18.5dpc MEF group nor were we able to classify these genes based on their cellular localization. Whereas in the 13.5dpc MEF, several pathways were significantly up-regulated and also the genes could be classified based on their cellular localization (Table 1). Further stringent filtering (p<0.05) of the categories based on cellular localization yielded the following list of pathways that were activated (Table 2). These genes were involved in various functions including cell adhesion, differentiation, proliferation, ECM remodeling, growth factor and regulation etc. Table 3 gives the details of the genes that belong to extra- cellular, secretory or cell surface associated categories.

The human transcriptome was studied based on the hybridization intensity. A total of 8111 genes showed hybridization intensity greater than 100 of which 2317 genes were highly expressed and showed intensity greater than 1000.

Comparative studies of microarray data with published proteomics data

Comparisons were based on fold changes in the corresponding eighty- five genes between 13.5dpc MEF and 18.5dpc MEF. Array hybridization intensities of HFF have been listed in Table 4. Upon comparisons, four expression patterns were observed: (i) certain genes were upregulated in both 13.5dpc MEF and HFF; (ii) certain genes were down regulated in both; (iii) upregulated in 13.5dpc MEF but not in HFF and (iv) some which were not upregulated in 13.5dpc MEF but were highly expressed in HFF.

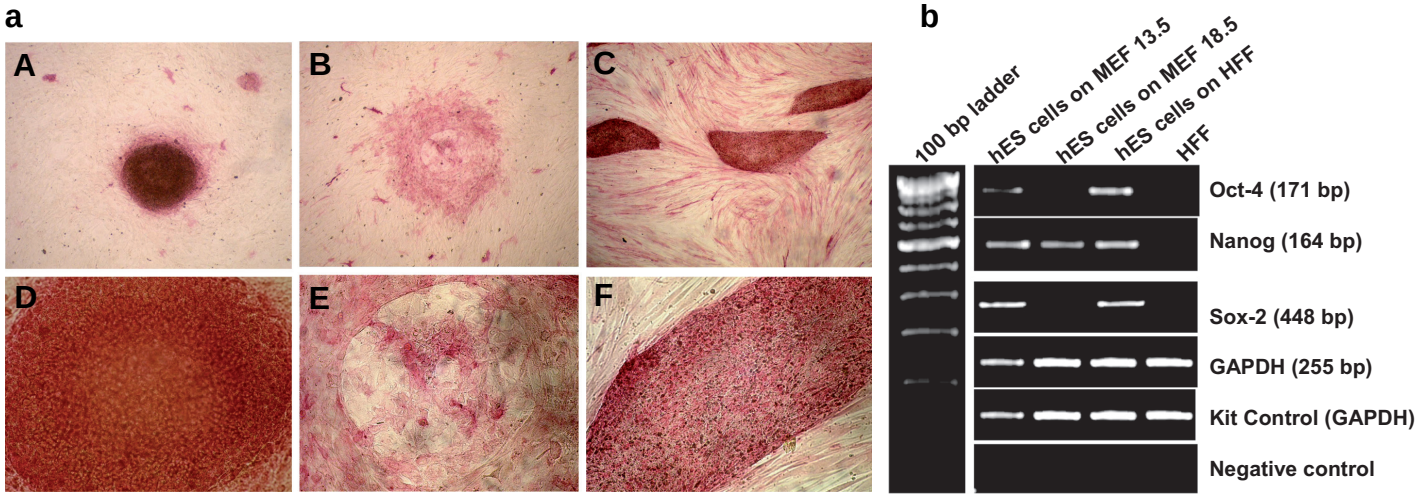

Fig. 1. Pluripotency assessment of hES cells grown on different feeder layers by alkaline phosphatase staining and RT-PCR. (a) *Alkaline phosphatase (AP) activity in hES cells growing on 13.5dpc MEF* **(A,D)**, *18.5dpc MEF* **(B,E)** *and on human feeders* **(C,F)**. *Note the marked absence of AP staining in the hES cells growing on 18.5dpc MEF* **(B,E)**. *The central part of the hES colony is differentiated and lacks AP positive cells. hES colonies growing on 13.5dpc MEF and HFF reveal positive staining for AP indicating absence of any sign of differentiation. Interestingly the human fetal fibroblasts (HFF) also reveal moderate AP activity (original magnification: A- C x40; D-F x100).* **(b)** *RT-PCR analysis of pluripotent marker expression in hES cells grown on various feeders and HFFs. Note the reduced expression of Oct-4, Nanog and Sox-2 in hES cell colonies grown on MEF 18.5 indicative of differentiation.*

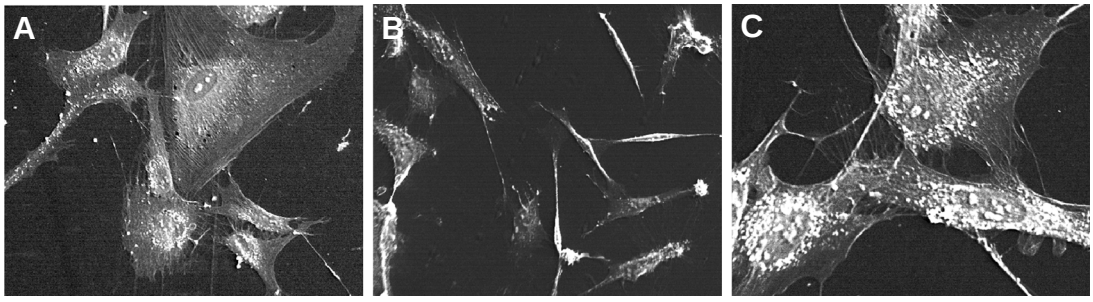

Fig. 2. Scanning electron micrographs of the feeder fibroblasts. **(A)** *13.5dpc MEF,* **(B)** *18.5dpc MEF and* **(C)** *HFF (original magnification x 500). Note the polygonal shaped myofibroblasts and the fibrils around the cell surface.*

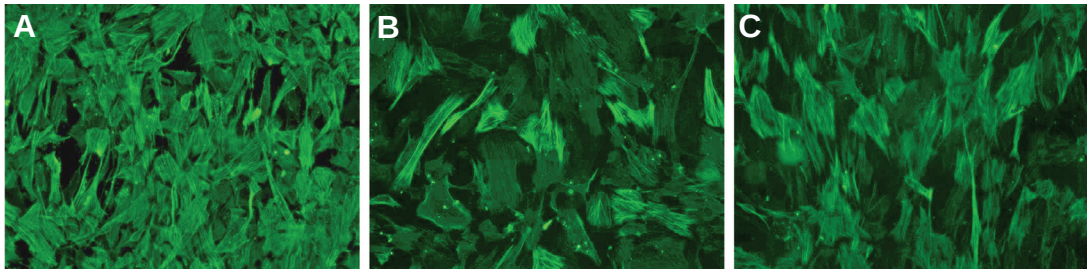

Fig. 3. Immunolocalization of α-SMA *in* **(A)** *13.5dpc MEF,* **(B)** *18.5dpc MEF and* **(C)** *HFF (original magnification x100)***.**

We worked on the premise that genes having a role in supporting hES cells proliferation *in vitro* should be upregulated in the supportive feeders. The proteins which were upregulated in both 13.5dpc MEF and HFF emerge as important candidates for further analysis and are indicated in bold (Table 4).

Scanning electron microscopy

Fibroblasts from 13.5dpc MEF and HFF appeared bigger in size, polyhedral and had several fibrils on their periphery which may be analogous to stress fibers described earlier (Tomasek *et al.,* 2002). The 18.5dpc MEF appeared more spindle shaped, quiescent and few polyhedral cells were observed but found to be devoid of peripheral fibrils (Fig 2)**.**

Immunocytochemical localization of α-SMA

α-SMA, a marker for myofibroblasts, was predominantly localized in large amounts in 13.5dpc MEF and HFF, as compared to the 18.5dpc MEF (Fig 3). As evident, the cells positive for α-SMA were polyhedral in shape and had bundles of fibrils staining positive.

Western blot analysis of α-SMA expression

Western blot and densitometry analysis showed the significant decrease in expression of α-SMA in non-supportive 18.5dpc MEF (Fig 4 A,B) as compared to 13.5dpc MEF. Human fibroblasts α-SMA expression, however, could not be normalized against GAPDH due to species variation as GAPDH is over expressed in HFF. However, the integrated optical density (IOD) of α-SMA for HFF was found to be the 1.30 and 1.04 fold higher than 18.5dpc MEF and 13.5dpc MEF respectively.

Discussion

A comparison of one biological system versus another is an interesting approach to study their differential functional status and biological properties since this will be reflected in their altered gene expression resulting in altered transcriptome and proteome. This approach was adopted in the present study with the hope to identify certain growth factors / proteins, secreted by the supporting feeder layers that may play a role in sustaining the undifferentiated proliferation of human embryonic stem cells *in vitro* and

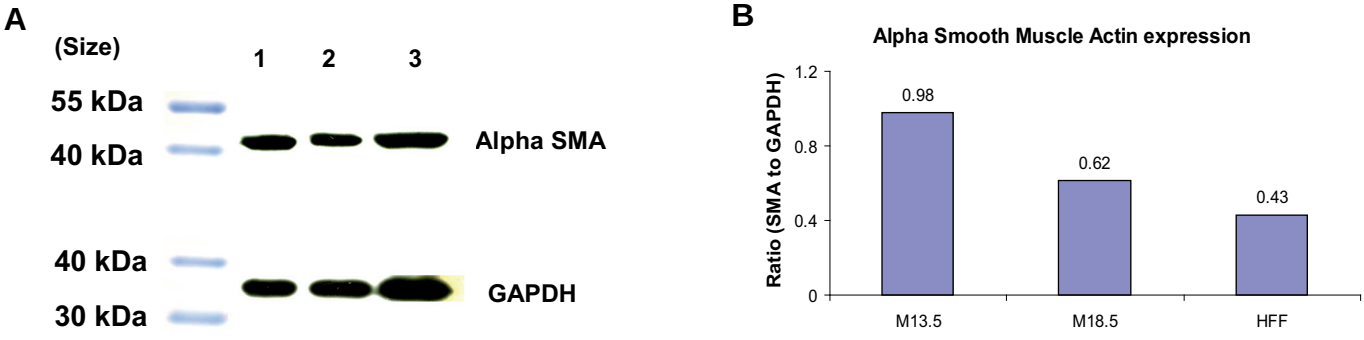

Fig. 4. Comparison of α SMA protein levels among different feeder fibroblasts. (A) *Western blot analysis of α-SMA expression in feeder fibroblasts: (1) 13.5dpc MEF, (2) 18.5dpc MEF and (3) HFF.* **(B)** *Quantitative analysis of α-SMA expression in feeder fibroblasts. Ratio= IOD alpha SMA/ IOD GAPDH; IOD, Integrated Optical Density.*

TABLE 1

CLUSTERING OF GENES UPREGULATED IN 13.5DPC *VS.* 18.5DPC MOUSE EMBRYONIC FIBROBLASTS

Clusters	No. of genes	P value
Pathway		
Glycan structures biosynthesis 1	4	1E-09
Cell Communication	6	0.0001
Apoptosis	2	0.0001
Cytokine-cytokine receptor interaction	8	0.0002
Chromosome		
14	13	0.0018
1	16	0.0033
Biological Functions		
Activation	77	1E-09
Ageing	14	1E-09
Angiogenesis	35	1E-09
Antigen Processing and Presentation	39	1E-09
Apoptosis	50	1E-09
Cell Adhesion	54	1E-09
Cell Differentiation	48	1E-09
Cell Division	32	1E-09
Cell Migration	26	1E-09
Cell Morphogenesis	31	1E-09
Cell Proliferation	53	1E-09
Chemotaxis	18	1E-09
Drug Response	19	1E-09
Endocytosis	14	1E-09
Extracellular Matrix Remodeling	35	1E-09
Gene Regulation	37	1E-09
Growth Factors And Regulators	68	1E-09
Immune Response	82	1E-09
Infection	29	1E-09
Inflammation	68	1E-09
Negative Gene Regulation	27	1E-09
Biosynthesis	23	0.0001
Degeneration	13	0.0002
Hemostasis	5	0.0007
Complement Activation	4	0.001
Anti-Apoptosis	6	0.0015
Metabolism	41	0.0025
Cell Cycle	24	0.0045
Homeostasis	15	0.0051
Cellular Localization		
Extracellular	62	1E-09
Secreted	33	1E-09
Plasma Membrane	32	0.0038

TABLE 2

SIGNIFICANT PATHWAYS IDENTIFIED FROM GENES LISTED IN CELLULAR LOCALIZATION CLUSTER (TABLE 1)

Functional Pathway	No. of genes	P value
Extra-cellular		
Cell Communication	4	1E-09
Cytokine-cytokine receptor interaction pathway	7	1E-09
MAPK signaling pathway	4	0.0044
Hematopoietic cell lineage	4	0.0082
Small cell lung cancer	4	0.0128
Complement and coagulation cascades	3	0.0486
TGF-beta signaling pathway	3	0.0486
Secretory		
Cytokine- cytokine- receptor interaction pathway	7	IE- 09
MAPK pathway	4	IE- 09
TGF beta signaling pathway	3	0.0001
Plasma Membrane		
Hematopoietic cell lineage	4	1E-09
Neuroactive ligand-receptor interaction	2	0.0014
Focal adhesion	3	0.0028
Propanoate metabolism	1	0.0128
Cell Communication	3	0.0374
B cell receptor signaling pathway	2	0.0474

may also facilitate feeder- free expansion of hES cells in future. The analysis was based on the hypothesis that any protein/ growth factor upregulated (>1.5 fold, log2) in supportive 13.5dpc MEF and HFF versus non-supportive 18.5dpc MEF feeders may play a crucial role in self- renewal of hES cells. It is also possible that MEF and HFF may act through different mechanisms but this possibility seems to be less likely.

Several pathways involved in cell proliferation, cell communication, cytokine- cytokine receptor interaction were found to be activated in 13.5dpc MEF transcriptome (Table 1-3) and interestingly while filtering these pathways based on cell localization in both extracellular and secreted milieu, TGFβ and associated signaling molecules emerged as strong candidates (Table 4). Several TGFβ and FGF2 associated proteins have been reported earlier in the conditioned medium (Prowse *et al.* 2007; Kueh *et al.* 2006) using proteomics approach. However, the absence of the peptide itself was attributed to the instrument limitation, protein abundance and size (Prowse *et al.* 2007). Results of the present study have led to the short listing of 37 out of reported 85 proteins (detected in high amounts in conditioned medium by proteomics approach) that may be involved in self- renewal of hES cells (Table 4). Interestingly, absence of activation of any well- defined cellular pathway involved in cell proliferation etc amongst the 43 genes that were 1.5 (log 2) fold higher ($p< 0.01$) in the 18.5dpc MEF (data not shown) perhaps explains why these feeder layers are non- supportive in nature and researchers prefer to use 13.5dpc embryos to derive feeder layers for hES cells cultures.

At the transcriptome level, an unambiguous up- regulation of TGFβ and associated molecules in 13.5dpc versus 18.5dpc MEF was evident, in agreement with published literature where TGFβ family has been implicated in hES cells proliferation (Diecke *et al.* 2008; Greber *et al.* 2007; Roberts *et al.,* 2005). The intracellular machinery of the supporting feeder layers appears to be geared up to stimulate the production of TGFβ and associated molecules (Table 4). Latent transforming growth factor binding protein (LTBP 1) was found to be differentially upregulated in supportive feeders. It is an extracellular matrix glycoprotein and plays a critical role in controlling and directing the activity of TGFβ by binding to latent TGFβ and thus regulating its availability and activity (Prowse *et al.* 2007). The three components of extra cellular matrix identified viz fibronectin, SPARC and thrombosondin have all been implicated in stimulating the production of TGFβ directly or through LTBP 1. Fibronectin is an extracellular matrix protein which has been implicated in the TGFβ signaling via assembly of LTBP1. SPARC is a glycoprotein that is also understood to modulate the action of growth factors including TGFβ. Thrombospondin is also known to activate TGFβ.

Regulation of hES cells self- renewal is a complex mechanism. Three key pathways implicated in hES cells differentiation, pluripotency and growth are Wnt, Inhibin and BMP/ TGFβ (Prowse *et al.* 2007). However, Wnt/ beta catenin activation does not appear to be important to maintain undifferentiated and pluripotent state

TABLE 3

CANDIDATE UPREGULATED GENES IN 13.5DPC MEF GROUPED ACCORDING TO CELLULAR LOCALIZATION

Unigene ID	Gene Name	Pathway Name
	Secretory	
Mm.8655	Complement component factor h	Complement and coagulation cascades
Mm.235105	Coagulation factor XIII, A1 subunit	Complement and coagulation cascades
Mm.42095	Secreted frizzled-related protein 4	Wnt signaling pathway
Mm.4339	Laminin, alpha 5	Focal adhesion
Mm.30211	Chemokine (C-X-C motif) ligand 14	Cytokine-cytokine receptor interaction
Mm.6813	Bone morphogenetic protein 4	TGF-beta signaling pathway
Mm.867	Chemokine (C-C motif) ligand 12	Cytokine-cytokine receptor interaction
Mm.8846	Fibroblast growth factor 9	MAPK signaling pathway
Mm.18213	Transforming growth factor, beta 2	TGF-beta signaling pathway Cytokine-cytokine receptor interaction MAPK signaling pathway
Mm.1293	Tumor necrosis factor	TGF-beta signaling pathway Cytokine-cytokine receptor interaction MAPK signaling pathway
Mm.303231	Chemokine (C-X-C motif) ligand 12	Cytokine-cytokine receptor interaction
Mm.303231	Chemokine (C-X-C motif) ligand 12	Cytokine-cytokine receptor interaction
Mm.244263	Chemokine (C-C motif) ligand 4	Cytokine-cytokine receptor interaction
Mm.1259	Nerve growth factor	MAPK signaling pathway;
Mm.3986	Growth hormone receptor	Jak-STAT signaling pathway Cytokine-cytokine receptor interaction
Mm.2423	Collagen, type II, alpha 1	Focal adhesion
Mm.193099	Fibronectin 1	Focal adhesion
Mm.103593	Dickkopf homolog 2 (Xenopus laevis)	Wnt signaling pathway
	Extracellular	
Mm.4339	Laminin, alpha 5	Cell Communication
Mm.30211	Chemokine (C-X-C motif) ligand 14	Cytokine-cytokine receptor interaction
Mm.6813	Bone morphogenetic protein 4	TGF-beta signaling pathway
Mm.867	Chemokine (C-C motif) ligand 12	Cytokine-cytokine receptor interaction
Mm.8846	Fibroblast growth factor 9	MAPK signaling pathway
Mm.18213	Transforming growth factor, beta 2	TGF-beta signaling pathway; Cytokine-cytokine receptor interaction; MAPK signaling pathway
Mm.1293	Tumor necrosis factor	TGF-beta signaling pathway; Cytokine-cytokine receptor interaction; MAPK signaling pathway
Mm.303231	Chemokine (C-X-C motif) ligand 12	Cytokine-cytokine receptor interaction
Mm.155586	Collagen, type IV, alpha 6	Cell Communication
Mm.244263	Chemokine (C-C motif) ligand 4	Cytokine-cytokine receptor interaction
Mm.1259	Nerve growth factor	MAPK signaling pathway
Mm.3986	Growth hormone receptor	Cytokine-cytokine receptor interaction
Mm.2423	Collagen, type II, alpha 1	Cell Communication
Mm.193099	Fibronectin 1	Cell Communication
	Plasma Membrane	
Mm.6712	Desmin	Cell Communication
Mm.227	Integrin alpha V	Focal adhesion
Mm.28095	Filamin, beta	Focal adhesion
Mm.193099	Fibronectin 1	Focal adhesion Cell Communication
Mm.390683	Gap junction protein, beta 2	Cell Communication

of hES cells (Dravid *et al.* 2005). Various researchers identified different molecules e.g. FGF 2 signaling, ECM proteins (Kueh *et al.* 2006), Noggin and FGF 2 (Xu *et al.* 2005) to be the key factors in promoting self- renewal of hES cells *in vitro*. Recently, FGF- 2 was demonstrated to act on MEF to release supportive factors including key members of the TGFβ pathway viz. inhibin alpha, TGFβ1, GREM-1, and BMP-4 and reduce differentiation-inducing activity (Greber *et al.* 2007). Interestingly, our results demonstrate an up regulation of key members of the TGFβ pathway even in the absence of FGF 2 in the supportive feeder transcriptome.

TGFβ has been implicated by several groups, in regulating the biology of embryonic stem cells and may help define the selection of cell fate and the progression of differentiation along a lineage (Mishra *et al.* 2005). We propose that when the hES cells are in close contact with the feeder layers, TGFβ facilitates proliferation and it is the microenvironment of ES cells that triggers TGFβ to exert contradictory roles of either facilitating proliferation of ES cells in presence of feeders or inducing differentiation when feeder support is withdrawn. Thus TGFβ acts through different pathways depending on the presence or absence of feeder support and appears to play dual role in both supporting differentiation and proliferation of hES cells. This hypothesis is further supported by an elegant study carried out by James and co-workers (2005) where they reported that in the undifferentiated hES cells**,** TGFβ /activin/nodal pathway is activated through the signal transducer SMAD2/3 and upon early differentiation SMAD1/5 signaling gets activated.

Embryonic stem cells share several characteristics of cancer cells including loss of contact inhibition and immortality (Gammill and Bronner, 2002). It is interesting to draw an analogy between cancer cells and surrounding stromal fibroblasts with embryonic stem cells and surrounding feeder fibroblasts. The complex and apparently contradictory role of TGFβ in cancer biology has been extensively studied where it shifts its role from an inhibitor to a promoter of proliferation during tumor progression (Ao *et al.* 2007; Elliott *et al.* 2005). TGFβ is understood to play a role in the communication between the cancer cells and the surrounding stromal cells. It regulates the interaction between cell and extracellular matrix through induction of extracellular matrix proteins such as fibronectin, collagen, and laminin etc. Several such ECM components were observed to be highly expressed in the supportive feeders (Table 2). The tissue stroma is understood to play an important role during cancer cell proliferation and invasion. The production of growth factors, chemokines and extracellular matrix by the surrounding stromal cells facilitate the malignant progression of cancer and represent an important target for cancer therapies (Wever *et al.* 2003; Kalluri *et al.* 2006, Billottet *et al.* 2008). It is widely accepted that the development of carcinoma is not only due to somatic mutations in epithelial cells but also is influenced by the tumor microenvironment including the stromal fibroblasts (Stover *et al.* 2007). Similarly for self- renewal of hES cells, feeder fibroblast layers are essential and when the feeder fibroblast support is withdrawn, results in differentiation of hES cells.

Stromal changes at the invasion front in a tumor (Vaughan *et al.,* 2000; Rice *et al.,* 2003) or during wound healing (Tomasek *et al.* 2002) include the appearance of myofibroblast cells, which are unique cells, an intermediate state between fibroblasts and smooth muscle cells (Gabbiani *et al.* 1971) that arise from transient differentiation of resident fibroblasts through multiple paracrine – mediated pathways including TGFβ (Shi-wen *et al.,* 2009). They are known to undergo mesenchymal-epithelial interactions and through the secretion of cytokines, chemokines, growth factors and extracellular matrix proteins, they facilitate cell growth and proliferation. To study similar phenomenon occurring in feeder layers, we examined the expression of myofibroblasts specific markers between the transcriptome of supportive and non- supportive feeders. Myosin heavy chain protein, vimentin and desmin were found to be 8, 2 and 21 fold up- regulated respectively.

Further assessment of morphological features and presence of α-SMA indicates that supportive feeder fibroblasts transiently differentiate into myofibroblasts and this decides whether a feeder layer is supportive or not for ES cells proliferation since the 18.5dpc MEF comprised predominantly of quiescent fibroblasts. Few polyhedral fibroblasts were also observed in 18.5dpc MEF corresponding to proto- myofibroblasts may be because of being cultured *in vitro* (Tomasek *et al.* 2002). We believe that similar to facilitating cancer cells proliferation, these cells also facilitate undifferentiated proliferation of ES cells. Thus it is proposed that a complex autocrine / paracrine network exists between feeder cells and ES cells involving the transient differentiation of myofibroblasts in the supportive feeders that upregulate ECM proteins and growth factors including TGFβ in the supportive feeder layers. Recently mechanical strain of myofibroblast was

TABLE 4

COMPARISON OF PUBLISHED PROTEOME DATA WITH TRANSCRIPTOME DATA OF SUPPORTIVE & NON-SUPPORTIVE FEEDER FIBROBLASTS

Proteins detected in the conditioned medium of supportive feeder fibroblasts taken from published literature	Fold change of hybridization intensity between 13.5 vs 18.5 dpc MEF	Hybridization intensity in HFF
Extra cellular Matrix & Remodeling Category		
Bone proteoglycan I(Biglycan)		±
Bone proteoglycan II (Decorin)	**2.56**	**++++**
Cathepsin K (pycnodysostosis)	**3.42**	**+++**
Collagen α 1(I)	1.05	0
Collagen alpha 1 (III)	**2.56**	**++**
Collagen α-1 (v)	1.03	+++
Collagen alpha 1 (VI)	0.30	±
Collagen alpha 1 (VIII)	1.00	++
Collagen alpha 1 (XII)	–	+++
Collagen alpha 1 (XIV)	–	–
Collagen alpha II (I)	0.99	++++
Collagen alpha II (V)	**2.05**	**++++**
Collagen alpha II (VI)	**2.35**	**+++**
Collagen alpha III (VI)	–	–
Hyaluronan and proteoglycan link protein 1	–	–
Hyaluronan synthase 2	9.41	–
Hephaestin	6.2	±
Heparin sulfate proteoglycan 2	–	–
Sulfatase 1	6.2	±
EGF containing fibulin like ECM protein – 1	**14.8**	**++++**
EMILIN-1	**3.02**	**++++**
Fibronectin 1	**2.74**	**++++**
Fibulin-1	**2.23**	**++++**
Fibulin-2	–	+++
Fibulin-5	1.41	+++
Heparan sulfate Proteoglycan Core Protein	**6.18**	**++**
Inhibin beta A (activin A, activin AB alpha peptide)	**4.49**	**++++**
Laminin gamma -1 chain	**4.67**	**+++**
Laminin α-4	**2.87**	**++++**
Laminin β-1	–	++++
Lumican	–	+++
Lysyl Oxidase-like-1	**2.92**	**++++**
Matrix Metalloproteinase-2	**2.72**	**++**
Metallo proteinase inhibitor-1 (TIMP-1)	**5.49**	**++++**
Metallo proteinase inhibitor-2(TIMP-2)	–	++++
Mimecan	–	–
Nidogen	–	–
Nidogen I	6.90	±
Nidogen II	**6.93**	**+++**
Osteoblast Specific Factor 2 (Periostin)	1.56	–
Plasminogen Activator inhibitor- 1	**6.01**	**+++**
Procollagen C-Endopeptidase Enhancer	1.28	++++
Protein-Lysine 6-oxidase	**5.94**	**++++**
SPARC	**2.13**	**++++**
Spondin 2	18.7	–
Stromelysin 1	**8.62**	**++++**

TABLE 4 (CONTINUED)

Proteins detected in the conditioned medium of supportive feeder fibroblasts taken from published literature	Fold change of hybridization intensity between 13.5 vs 18.5 dpc MEF	Hybridization intensity in HFF
Tenascin C (Hexabrachion)	–	–
TGF-β induced protein IG-H3	–	–
Thrombospondin-1	**5.60**	**+++**
Versican core protein	–	–
Role in formation & regulation of cytoskeleton		
Actin beta	2.18	++++
Actin binding LIM protein 1	–	+++
Filamin A	–	+++
Gelsolin	1.58	++++
Lamin A/C	–	++++
Laminin alpha 5	**25.2**	**+**
Myosin Heavy Chain Peptide 9 (H) Myosin Heavy Chain Peptide 10 (M)	**5.96**	**++++**
Myosin Light Chain	–	–
Profilin 2	9.62	–
Profilin 1	**2.62**	**++++**
Transgelin 2	–	–
Tubulin beta 1	–	–
Vimentin	**1.9**	**++++**
Viniculin	–	–
Growth Factors		
Activin A (Inhibin)	–	+++
BMP 1	2.91	±
BMP 4	18.1	±
Actinin, alpha 4	–	–
Dickkopf homolog-3	**5.25**	**+++**
FGF 2	–	++
Follistatin Related Protein-1 (FRP 1)	–	–
Gremlin-1	**1.8**	**++++**
IGFBP 1	–	–
IGFBP 2	**8.35**	**++++**
IGFBP 3	2.1	–
IGFBP 4	**3.48**	**++++**
IGFBP 5	–	+++
IGFBP 6	**3.59**	**+++**
IGFBP 7	**2.00**	**++++**
Insulin like growth factor 1	4.97	–
Insulin like growth factor 2	**2.07**	**+++**
Latent Transforming Growth Factor-β Binding Protein isoform 1 (LTBP-1)	**8.26**	**+++**
LTBP 3	5.06	–
Pigment Epithelium derived factor	–	++++
STAT 3	4.7	±
TGF alpha	–	–
TGF beta 1	**2.9**	**+**
TGF beta 1 induced transcript4	6.28	–
Vasorin	–	–
ADAM 11		–

Hybridization intensity in human array >100 has been designated; between 100- 200 as +; between 200- 500 as +; between 500- 1000 as ++; between 1000- 5000 as +++ and > 5000 as ++++).
Upregulated proteins in supportive feeders are marked in bold in the text.

reported to induce autocrine or paracrine signaling through TGFβ super- family ligands that supports hES cells self- renewal (Saha *et al.* 2008). Further studies are ongoing in this direction to explore the phenomenon still better.

Materials and Methods

Cell culture

Human fetal fibroblast (HFF) culture

Human fetal fibroblast culture, with prior permission from Institute Ethics Committee, was established as described earlier (Kumar *et al.,* 2009).

Mouse embryonic fibroblast (MEF) culture

13.5 and 18.5dpc CF-1 mouse embryos were washed three times with phosphate buffer saline (PBS) containing 1% v/v penicillin-streptomycin. Using sterile scissors limbs, head, tail and dark red organs were removed. Remaining body parts were finely minced in 0.5% Trypsin EDTA (Sigma, St Louis, MO) and incubated at 37^0C for 20 min. Tissues were then mixed with equal volume of fetal bovine serum (HyClone, South Logan, UT) to stop the activity of Trypsin EDTA. Cells were released by triturating gently and centrifuged at 1000 rpm for 5min. Pellets were then resuspended in to media containing DMEM high glucose (Gibco/ Invitrogen, Carlsbad, CA), 10% FBS, 1%v/v L-Glutamine (Gibco), 1% v/v non essential amino acids (Sigma) and 1% v/v Pen/strep (Sigma) and incubated in T-75 flasks (Nunc, Rochestor, NY) at 37^0C & 5% CO_2. Cells were monitored for their growth and sub-cultured regularly. For passaging, confluent cultures were washed with PBS and then incubated with 0.05% Trypsin EDTA for 4 -5 min. The pellet was re-suspended in to MEF media and incubated at 37^0C & 5% CO_2. Cells grown up to passage 3 from 8- 10 flasks were pooled together and used for further studies.

Microarray analysis

Transcriptional changes between 13.5 and 18.5dpc MEF were studied using Agilent Mouse Genome 8x15k array (AMADID 16270) starting with total RNA. Human fetal fibroblasts were similarly analyzed using human genome 8x15k array (AMADID 16332). All the three arrays were done using single color microarray based gene expression analysis.

RNA extraction and quality control

For RNA extraction, cells were suspended in RNAlater (Qiagen, USA)) immediately after harvesting at room temperature. After permeabilisation of RNAlater for at least an hour, total RNA was isolated from the cells using RNAeasy Mini kit (Qiagen, USA) according to the instructions of the manufacturer. RNA integrity was assessed using RNA 6000 Nano Lab Chip on the 2100 Bioanalyzer (Agilent, USA) following the manufacturer's protocol. Total RNA purity was assessed by the NanoDrop Spectrophotometer (Nanodrop Technologies, USA). Total RNA with OD260/ OD280>1.8 and OD260/OD270 ≥ 1.3 was used for microarray experiments.

cRNA synthesis, labeling and microarray hybridization

Low RNA Input Fluorescent Linear Amplification Kit (Agilent, CA) was used for labeling. Briefly, both first and second strand cDNA were synthesized by incubating 500ng of total RNA with 1.2µl of oligo dT-T7 Promoter Primer in nuclease-free water at 65^0C for 10 min followed by incubation with 4.0µl of 5◊ First strand buffer, 2µl of 0.1M DTT, 1 µl of 10mM dNTP mix, 1µl of 200 U/µl MMLV-RT, and 0.5µl of 40U/µl RNaseOUT, at 40^0C for 2 hour. Immediately following cDNA synthesis, the reaction mixture was incubated with 2.4 µl of 10 mM Cyanine-3-CTP (Perkin-Elmer, MA), 20µl of 4X Transcription buffer, 8 µl of NTP mixture, 6 µl of 0.1M DTT, 0.5 µl of RNaseOUT, 0.6µl of Inorganic pyrophosphatase, 0.8 µl of T7 RNA polymerase, and 15.3µl of nuclease-free water at 40^0C for 2 hour. Qiagen's RNeasy mini spin columns were used for purifying amplified samples. The quantity and specific activity of cRNA was determined by using NanoDrop spectrophotometer. Samples with specific activity >8 were used for hybridization. 825ng of each Cyanine 3 labeled cRNA in a volume of 41.8µl were combined with 11µl of 10x blocking agent and 2.2µl of 25x fragmentation buffer, and incubated at 60^0Cfor 30 minutes in dark. The fragmented cRNA were mixed with 55µl of 2x hybridization buffer. About 10µl of the resulting mixture was applied to the 8x15k Gene Expression Microarray (Agilent Technologies), and hybridized at 65^0C for 17 hours. After hybridization, slides were washed with wash buffer for 1 min at room temperature followed by a 1 min wash with wash buffer II at 37^0C. Slides were finally rinsed with acetonitrile for cleaning up and drying.

Scanning & feature extraction

Hybridized arrays were scanned at 5µm resolution on a DNA scanner and data extraction from images was done using Feature Extraction software.

Data analysis and biological interpretation

Microarray output images were manually examined for excessive noise and physical anomalies. Feature extracted data was analyzed using GeneSpring GX version 7.3.1 (Genotypic, Bangalore, India) and Microsoft Excel software. Normalization of the data was done within GeneSpring using the recommended per chip and per gene data transformation which included the set measurement less than 0.01 to 0.01, per chip normalization to 50th percentile and normalization of each gene to the median. Further quality control of normalized data was done using correlation based condition tree to eliminate bad experiments. In order to assess the differentially expressed genes between 13.5 and 18.5dpc MEF, up and down regulated genes were subjected to functional analysis using GeneSpring GX software ontology browser by selecting appropriate parameters in the tool. The statistically significant transcripts were annotated using web based Biointerpreter version 1.1 (Genotypic) software to generate clusters of functionally related genes to understand their biological significance.

Comparative studies of microarray data with published proteomics data

A total of eighty-five proteins reported earlier in the conditioned medium of supportive feeder layers (Lim *et al.* 2002; Prowse *et al.* 2005*,* 2007; Kueh *et al.* 2006; Eiselleova *et al.* 2008) and belonging to the categories of extracellular matrix proteins, cell cytoskeleton and growth factors, were analyzed at the transcriptome level between supportive (13.5dpc MEF and HFF) and non- supportive (18.5dpc MEF) feeders.

Scanning electron microscopy

Fibroblast cells were grown on sterile 100mm^2 glass coverslips. They were fixed in 2.5% glutaraldehyde (Pelco International, Redding, CA) in 0.1M cacodylate (Pelco) buffer (pH 7.4), after two washes in phosphate buffer saline (PBS) to remove media and serum contents. After several washes in the cacodylate buffer, the cells were post fixed with buffered 1.0% osmium tetroxide (Pelco) for 30min at 4^0C. Later the coverslips were washed in buffer, dehydrated in a ascending series of ethanol and critical point dried for 20 min in liquid CO_2 in a E 3100 critical point drier (Quorum technologies; www.quorumtech.com, UK). The coverslips were then stuck to aluminum stubs and coated with gold-palladium for 20 sec at 20 mA using Polaron sputter coater (SC7640, Quorum Technologies) coating apparatus. Fibroblasts were then examined under JEOL 6400 scanning electron microscope (JEOL, Tokyo, Japan) and representative areas were photographed.

Immunocytochemical localization of α-smooth muscle actin (α–SMA)

Adherent fibroblast cells on coverslips were fixed with 4% paraformaldehyde in PBS (Sigma) for 10 min and permeablized with 0.1% Triton-X 100 for 5 min. Cells were washed, blocked with 3% BSA (Sigma) and

later incubated overnight with monoclonal αSMA antibody (Dako, Glostrup, Denmark) at 4^0C. AlexaflourTM 488 (Molecular Probe, Invitrogen) conjugated secondary antibody diluted 1: 1000 in blocking solution was used to visualize the specific localization of αSMA antibody in supportive and nonsupportive fibroblasts. Representative areas were photographed under florescent microscope (90i, Nikon, Japan).

Western Blot analysis

Feeder fibroblasts of 13.5dpc MEF, 18.5dpc MEF and HFF, cultured in T-75 flasks were harvested into 500 ul of 1X lysis solution (10mM Tris, pH 7.6, 100mM Nacl, 1mM EDTA, 1% v/v Triton X 100, 10% v/v Glycerol) supplemented with 1% v/v, 1X protease inhibitor mix (Amersham, GE Healthcare). 20ug of protein lysate was electrophoresed on 12% SDS-PAGE gel followed by electroblotting on PVDF membrane (Amersham, GE Healthcare). Membrane was then blocked with 5% NFDM in TBST and incubated with monoclonal primary mouse anti α-SMA antibody (1:1500) (Chemicon International, CA, USA) at 4^0C over night. Detection of α-SMA was performed by incubating membrane with HRP conjugated goat anti mouse secondary antibody (1:10,000) (Jackson Immuno Research Laboratories) for 2 hrs at room temperature followed by chemiluminescent (SuperSignal West Pico, Pierce, USA) exposure of Hyperfilm™ ECL (Amersham, GE Healthcare). Monoclonal mouse anti GAPDH antibody (1:5000) (Calbiochem, USA) was used as house keeping to quantitate alpha SMA Expression in various fibroblasts using Gene Tools software (Syngene, UK).

Acknowledgements

This study (BTPR3985/MED/14/49712003) was financially supported by Department of Biotechnology, Govt. of India. Authors are grateful to Prof Jayesh Bellare, Sillicate Lab, Indian Institute of Technology, Mumbai, for assisting in Scanning Electron Microscopy studies.

References

BILLOTTET, and JOUANNEAU, J. (2008). Tumor-stroma interactions. *Bull Cancer* 95: 51-56.

DE WEVER, O. and MAREEL, M. (2003). Role of tissue stroma in cancer cell invasion. *J Pathol* 200: 429-447.

DIECKE, S., QUIROGA-NEGREIRA, A., REDMER, T. AND BESSER, D. (2008) FGF2 signaling in mouse embryonic fibroblasts is crucial for self-renewal of embryonic stem cells. *Cells Tissues Organs* 188: 52-61.

DRAVID, G., YE, Z., HAMMOD, H., CHEN, G., PYLE, A., DONOVAN, P., YU, X. and CHENG, L. (2005). Defining the role of Wnt/ β-catenin signaling in the survival, proliferation, and self renewal of human embryonic stem cells. *Stem Cells* 23: 1489-1501.

EISELLEOVA, L., PETERKOVA, I., NERADIL, J., SLANINOVA, I., HAMPL, A. and DVORAK, P. (2008). Comparative study of mouse and human feeder cells for human embryonic stem cells. *Int J Dev Biol* 52: 353-363.

ELLIOTT, R.L. and BLOBE, G.C. (2005). Role of transforming growth factor beta in human cancer. *J Clin Oncol* 23: 2078-2093.

GABBIANI, G., RYAN, G. and MAJNE, G. (1971). Presence of modified fibroblast in granulation tissue and their possible role in wound contraction. *Experimentia* 27: 549-550.

GAMMILL, L.S., and BRONNER-FRASER, M. (2002). Genomic analysis of neural crest induction. *Development* 129: 5731-5741.

GREBER, B., LEHRACH, H. and ADJAYE, J. (2007). Fibroblast growth factor 2 modulates transforming growth factor b signaling in mouse embryonic fibroblasts and human ESCs (hESCs) to support hESC self renewal. *Stem Cells* 25: 455-464.

JAMES, D., LEVINE, A.J., BESSER, D. and HEMMATI-BRIVANLOU, A. (2005). TGFβ/activin/nodal signaling is necessary for the maintenance of pluripotency in human embryonic stem cells. *Development* 132: 1273-1282.

KALLURI, R. and ZEISBERG, M. (2006). Fibroblasts in cancer. *Nat Rev Cancer* 5: 392-401.

KUEH, J., RICHARDS, M., SZE-WAI, N.G., CHAN, W.K. and BONGSO, A. (2006). The search for factors in human feeders that support the derivation and propagation of human embryonic stem cells: preliminary studies using transcriptome profiling by serial analysis of gene expression. *Fertil Steril* 85: 1843-1846.

KUMAR, N., HINDUJA, I., NAGVENKAR, P., PILLAI, L., ZAVERI, K., MUKADAM, L., TELANG, J., DESAI, S., MANGOLI, V., MANGOLI, R., PADGAONKAR, S., KAUR, G., PURI, C. and BHARTIYA, D. (2009). Derivation and characterization of two genetically unique human embryonic stem cell lines on in-house derived human feeders. *Stem Cells Dev* 18:435-445.

LIM, J.W.E. and BODNAR, A. (2002). Protemome analysis of conditioned medium form mouse embryonic fibroblast feeder layers which support the growth of human embryonic stem cells. *Proteomics* 2: 1187-1203.

M, A.O., FRANCO, O.E., PARK, D., RAMAN, D., WILLIAMS and HAYWARD, S.W. (2007). Cross-talk between paracrine-acting cytokine and chemokine pathways promotes malignancy in benign human prostatic epithelium. *Cancer Res* 67: 4244-4253.

MARTIN, M.J., MUOTRI, A., GAGE, F. and VARKI, A. (2005). Human embryonic stem cells express an immunogenic nonhuman sialic acid. *Nat Med* 11: 228–232.

MISHRA, L., DERYNCK, R. and MISHRA, B. (2005). Transforming growth factor-β signaling in stem cells and cancer. *Cell Signaling* 310: 68-71.

PROWSE, A.B., MC QUADE, L.R., BRYANT, K.J., DYK, D.D., TUCH, B.E. and GRAY, P.P. (2005). A proteome analysis of conditioned medium from mouse embryonic fibroblast feeder layers which support the growth of human embryonic stem cells. *Proteomics* 5: 978-989.

PROWSE, A.B., MC QUADE, L.R., BRYANT, K.J., MARCAL, H. and GRAY, P. (2007). Identification of potential pluripotency determinants for human embryonic stem cells following proteomic analysis of human and mouse fibroblast conditioned media. *J Proteome Res* 6: 3796-3807.

RICE, N.A. and LEINWAND, L.A. (2003). Skeletal myosin heavy chain function in cultured lung myofibroblasts. *J Cell Biol* 163: 119-129.

RICHARDS, M., TAN, S., FONG, C.Y., BISWAS, A., CHAN, W.K. and BONGSO, A (2003). Comparative evaluation of various human feeders for prolonged undifferentiated growth of human embryonic stem cells. *Stem Cells* 21: 546-556.

ROBERTS, A. and MISHRA, L. (2005). Role of TGF-β in cells and cancer. *Oncogene* 24: 5667.

SAHA, S., JI, L., DE PABLO, J.J. and PALECEK, S.P. (2008). TGF beta/Activin/Nodal pathway in inhibition of human embryonic stem cell differentiation by mechanical strain. *Biophys J* 94: 4123-4133.

SHI-WEN, X., PARAPURAM, S.K., PALA, D., CHEN, Y., CARTER, D.E., EASTWOOD, M., DENTON, C.P., ABRAHAM, D.J. and LEASK, A. (2009). Requirement of transforming growth factor beta-activated kinase 1 for transforming growth factor beta-induced alpha-smooth muscle actin expression and extracellular matrix contraction in fibroblasts. *Arthritis Rheum* 60: 234-241.

STOVER, D.G., BIERIE, B. and MOSES, H.L. (2007). A delicate balance: TGF-b and the tumor microenvironment. *J Cell Biochem* 101: 851-861.

TOMASEK, J.J., GABBIANI, G., HINZ, B., CHAPONNIER, C. and BROWN, R.A. (2002). Myofibroblasts and mechanoregulation of connective tissue remodeling. *Nat Rev Mol Cell Biol* 3: 349-363.

VAUGHAN, M.B., HOWARD, E.W. and TOMASEK, J.J. (2000). Transforming growth factor-beta1 promotes the morphological and functional differentiation of the myofibroblast. *Exp Cell Res* 257: 180-189.

XU, R.H., PECK, R.M., LI, D., FENG, X., LUDWIG, T. and THOMSON, J.A. (2005). Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. *Nat Methods* 2: 185-190.

Further Related Reading, published previously in the *Int. J. Dev. Biol.*

See Special Issue ***Pattern Formation*** edited by Michael K. Richardson and Cheng-Ming Chuong at:
http://www.ijdb.ehu.es/web/contents.php?vol=53&issue=5-6

Comparative study of mouse and human feeder cells for human embryonic stem cells
Livia Eiselleova, Iveta Peterkova, Jakub Neradil, Iva Slaninova, Ales Hamp and Petr Dvorak
Int. J. Dev. Biol. (2008) 52: 353-363

Common culture conditions for maintenance and cardiomyocyte differentiation of the human embryonic stem cell lines, BG01 and HUES-7
Chris Denning, Cinzia Allegrucci, Helen Priddle, Maria D. Barbadillo- Muñoz, David Anderson, Tim Self, Nigel M. Smith, C. Tony Parkin and Lorraine E. Young
Int. J. Dev. Biol. (2006) 50: 27-37

The stroma reaction myofibroblast: a key player in the control of tumor cell behavior
Alexis Desmoulière, Christelle Guyot and Giulio Gabbiani
Int. J. Dev. Biol. (2004) 48: 509-517

Functional analysis of the TGFbeta receptor/Smad pathway through gene ablation in mice
M J Goumans and C Mummery
Int. J. Dev. Biol. (2000) 44: 253-265

Type beta transforming growth factors and activins in differentiating embryonal carcinoma cells, embryonic stem cells and early embryonic development
C L Mummery and A J van den Eijnden-van Raaij
Int. J. Dev. Biol. (1993) 37: 169-182

5 yr ISI Impact Factor (2009) = 3.253