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Comparative Analysis of Five Different Homologous Feeder Cell Lines in the Ability to Support Rhesus Embryonic Stem Cells

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Abstract: In our previous study, five homologous feeder cell lines, Monkey ear skin fibroblasts (MESFs), clonally derived fibroblasts from the MESFs (CMESFs), monkey oviductal fibroblasts (MOFs), monkey follicular granulosa fibroblast-like (MFGs) cells, monkey follicular granulosa epithelium-like (MFGEs) cells, were developed for the maintenance of rhesus embryonic stem cells (rESCs). We found that MESFs, CMESFs, MOFs and MFGs, but not MFGEs, support the growth of rhesus embryonic stem cells. Moreover, we detected some genes that are upregulated in supportive feeder cell lines by semi-quantitative PCR. In the present study, we applied the GeneChip® Rhesus Macaque Genome Array of Affymetrix Corporation to study the expression profiles of these five feeder cell lines, in purpose to find out which cytokines and signaling pathways were important in maintaining the rESCs. mRNAs of eight genes, including GREM2, bFGF, KITLG, DKK3, GREM1, AREG, SERPINF1 and LTBP1, were found to be upregulated in supportive feeder cell lines, but not in MFGE. The results indicate that many signaling pathways may play redundant roles in supporting the undifferentiated growth and maintenance of pluripotency in rESCs.

Key words: Embryonic stem cells; Rhesus monkey feeders; Self-renewal; Pluripotency; Expression profile; Signaling pathway

五株同源饲养层支持猕猴胚胎干细胞能力的比较

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摘要：我们以前的研究建立了五株猕猴饲养层细胞系来支持猕猴胚胎干细胞(rESCs)的生长：一岁猴耳皮肤成纤维细胞(MESFs)、两岁猴输卵管成纤维细胞(MOFs)、成年猴卵泡颗粒成纤维样细胞(MFGs)、成年猴卵泡颗粒上皮样细胞(MFGEs)，以及 MESFs 的克隆成纤维细胞(CMESFs)。我们发现 MESFs、CMESFs、MOFs 和 MFGs，而不是 MFGEs 支持猕猴胚胎干细胞(rESCs, rhesus embryonic stem cells)的生长。通过半定量 PCR 的方法，我们在支持性的饲养层细胞中检测到了一些基因的高表达。在本研究中，我们运用 Affymetrix 公司的 GeneChip® Rhesus Macaque Genome Array 芯片来研究这五株同源饲养层的表达谱，希望发现哪些细胞因子和信号通路在维持 rESCs 中起到重要作用。结果表明，除 MFGE 外，包括 GREM2、 bFGF、KITLG、DKK3、GREM1、AREG、SERPINF1 和 LTBP1 等八个基因的 mRNA 在支持性的饲养层细胞中高表达。本研究结果提示，很多信号通路在支持 rESCs 的未分化生长和多潜能性方面可能起到了冗余的作用。

关键词：胚胎干细胞；猕猴饲养层；自我更新；多潜能性；表达谱；信号通路

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Embryonic stem cells (ESCs) can proliferate indefinitely, maintain an undifferentiated state, and differentiate into any cell type when environmental signals change (Smith, 2001). Differentiation of ESCs into specific cell types may provide resources to cure or alleviate the symptoms of various degenerative diseases (Smith, 1998).

With the possibility of these cells becoming cancerous, a prudent approach towards advancing ESCs into human clinical trials is required. While the vast majority of animal research is conducted in rodents, we believe that the unanswered questions regarding ESCs *in vivo* function, apoptosis, and tumor formation would be best addressed using a non-human primate (NHP). Rhesus macaques (the standard nonhuman primate model organism) possess remarkable anatomical, physiological, and metabolic similarities to humans. Moreover, many human neurological diseases, such as Alzheimer and Parkinson diseases, can only be accurately modeled in the nonhuman primate. In addition to the biological similarities between rhesus macaques and humans, significant numbers of primate ESC lines are now available (Mitalipov et al, 2006), and ESCs derived from the rhesus monkey and human blastocysts demonstrate extensive similarities that are not observed in murine ESCs (Amit et al, 2000; Donovan & Gearhart, 2001; Ginis et al, 2004). This leads to the conclusion that rhesus monkey provides an accurate model for developing strategies to test the feasibility, safety, and efficacy of ESC-based medical treatments.

Despite the significance of rhesus monkey stem cells as a model for ESC-based medical treatments, little is known about the niche signals that maintain the self-renewal and pluripotency of rhesus stem cells. The microarray is a powerful new tool that can simultaneously analyze the expression of thousands of genes. Previous research has used microarray to investigate the transcriptional profile of feeder cells that support the undifferentiated state of human embryonic stem cells (Villa-Diaz et al, 2008). However, transcriptional profile analysis has not been conducted to study the niche signals that control the undifferentiated state of rhesus embryonic stem cells up to date. Performing transcriptional profile of feeder cells would provide important information on the external signals needed to maintain rhesus ESCs in a pluripotent state, demonstrate the similarities and differences between human and non-human primate ESCs, and provide a foundation for future pre-clinical ESC research and large-scale culture of non-human primate ESCs. The GeneChip Rhesus Macaque Genome Array uses 52,865 probe sets to detect the expression of over 47,000 transcripts, including transcripts with poly(A) sites (Duan et al, 2007), thus represents a powerful tool to study the transcriptional profiles of feeder cells.

In our previous study (Li et al, 2005), we developed five rhesus monkey feeder cell lines: the ear skin fibroblasts from a neonatal, 1-week-old monkey (monkey ear skin fibroblasts, MESFs), oviductal fibroblasts from a juvenile (2-year-old) animal (MOFs), adult follicular granulosa fibroblast-like (MFG) cells, adult follicular granulose epithelium-like (MFGE) cells, and clonally derived fibroblasts from the MESFs (CMESFs). These cell lines were tested for their ability to support the culture and propagation of rESCs, compared with MEFs, and we found that except for the MFGE cell line, all other cell lines were as good as or better than MEF feeder cells. The expression of several genes related to ESC growth, maintenance, and self-renewal was also checked, finding that LIF, CNTF, bFGF, SCF and BMP4 were upregulated in supportive feeder cell lines.

However, which cytokines and signaling pathways control the unlimited proliferation and pluripotency of rESCs remains largely unknown. In order to unravel this mystery, we applied the oligonucleotide array to analyze the expression profiles of the five rhesus fibroblast cell lines and quantitative real-time PCR (qPCR) to confirm the expression of several genes.Through this method the differentially expressed genes between the supportive and non-supportive feeder cell lines were expected to be determined. We investigated the cytokines located in the extracellular region or on the membrane of fibroblast cells, which may interact with rESCs through membrane-binding receptors and trigger the downstream signaling events that finally prolonged the undifferentiated growth of rESCs. Eight genes, GREM2, bFGF, KITLG, DKK3, GREM1, AREG, SERPINF1 and LTBP1, were found to be overexpressed in supportive feeder cells, and three of them, SCF, bFGF and GREM2, were validated by qPCR.

1 Materials and Methods

1.1 Culture media

Fibroblast culture medium was Dulbecco's modified Eagle's medium (DMEM) (Gibco, Invitrogen Corporation, Carlsbad, CA, http://www.invitrogen.com) supplemented with 10% newborn bovine serum (Gibco) containing 1× penicillin-streptomycin. Granulosa cells were cultured in the same medium with that of fibroblast cells. R366.4 rESCs (provided by Dr. James Thomson) were cultured in DMEM, 1 mmol/L glutamine (Sigma), 0.1 mmol/L mercaptoethanol (Sigma), 1×penicillin-streptomycin and 10% defined fetal bovine serum (Hyclone).

1.2 Feeder cells and rESCs culture

Feeder cells were aliquoted after amplification. After defreezing, feeder cells were cultured in fibroblast culture medium. We treated the feeder cells with 7.5 μg/mL mytomycin for 2.5 hours before using. rESCs, initially cultured on MEFs (Thomson et al, 1995), were plated onto homologous cell monolayers (passage 8 [P8] and mitotically inactivated by mytomycin). Once rESCs colonies grew to maximal size without visible differentiation, cells were digested with 10 mg/mL dispase (Gibco) and seeded onto newly prepared feeder cells.

1.3 RNA preparation and microarray analysis

Total RNA was extracted using a TRIZOL RNA isolation kit (Invitrogen Corporation). Potential contamination from genomic DNA was eliminated by RNase-free DNase (Invitrogen) digestion. The MIAME (Minimum Information About a Microarray Experiment) for microarray were imcorporated into the design and implementation of this study. The RNA samples were measured using the NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE). Labeling, hybridization, and scanning were performed according to standard Affymetrix protocols (see Affymetrix GeneChip Expression Analysis Technical Manual, http://www. affymetrix.com). The normalized microarray data were further analyzed using GeneChip Operating System (GCOS) 1.2. Gene expression fold changes between two samples were calculated from the following formula: $FC=2^{SLR}$ (FC: fold-changes, SLR: signal log ratio). The following selection criteria were used to identify candidate supportive genes: 1) the expression calls of individual probe sets should be considered to be present; 2) gene expression fold changes between MESFs, CMESFs, MFGs, MOFs and MFGEs should be more than 2. Once raw candidates were recieved, any duplicate probe sets were removed as some probe sets represent the same genes. Due to the study focusing on the genes products that functioned on rESCs, only genes encoding products that were located in the extracellular region or on the membrane of fibroblast cells were reserved for further analysis. A manual filtration through PubMed was applied. Finally, candidate genes were referred to as the "supporting-relative genes" (Fig. 1).

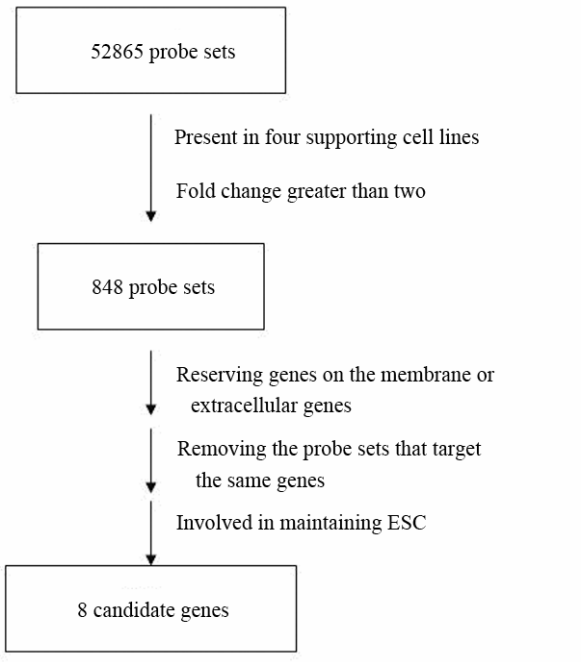

Fig. 1 Experimental design flow chart

1.4 Quantitative PCR

Total RNA was extracted from feeder cells in a T25 flask through QIAGEN RNeasy® Micro kit. RNA from each feeder cell line was treated with RNase-free DNase (Invitrogen) to remove genomic DNA contamination. 1μg of total RNA were reverse transcribed by SuperScript III (Invitrogen) using oligo(dT) primers and 1 μL of cDNA was used for quantitative PCR. Quantitative PCR was performed on MJ Opticon II with iQ SYBR Green Supermix (Bio-rad), and GAPDH was used as housekeeping gene. Primers are listed below. GAPDH: 5'-TGAAGGTCGGAGT- CAACGGA-3' (sense) and 5'-TGGTGCAGGAGGCATTGCTG-3' (antisense); SCF: 5'-TAAACGGAGTCGCCACACCAC-3' and 5'-GATTTGCCACCAATTTAGTGACG-3'; bFGF: 5'-CTATGCTGTGGAAGCACCGGA-3' and 5'-CCA-TCTCGAGGTGGGTCTCCC-3'; GREM2: 5'-TCATCT-TCGTTTATTAAATGGGAC-3' and 5'-ATT CATTAA-

ATAAGCTTTCTGGGA-3'.

2 Results

2.1 Prolonged expansion of rESCs cultured on rhesus monkey feeders

From the previously established cell lines of three MESF, two MOF, two MFG, two MFGE, and six CMESF , we randomly chose one cell line for each kind as feeders. rESCs were able to grow on MESFs, MOFs, CMESFs, and MFGs for 15–20 passages without differentiation. The rESCs were passaged every 7 days, similar to that normally required for cells on MEFs. In contrast, rESCs could not survive when cultured on the MFGE cell line.

2.2 High quality of RNA for microarray and quality assessment of microarray data

Obtaining the gene expression profile of biological samples by Affymetrix GeneChip microarrays is an elaborate process, in which each step may bring variation to the result of the microarray. A denaturing agarose gel electrophoresis was used for checking the RNA quality. The results revealed that the RNA samples of feeder cells were very clear-cut and bright, with the brightness of 28S to that of 18S around 2:1 and A_{260} to A_{280} about 2.0. All these results demonstrated that the quality of RNA is high enough for microarray experiments (Tab. 1).

In addition, the microarray process is both costly and time-consuming. Therefore, it is critical to utilize the information produced by the arrays and ascertain its quality. Comparing the distribution of probe intensities across all arrays can sometimes indicate that one array is unlike the others. Such an example is shown in Fig. 2, where all five sets of data remain nearly on the same level indicating the overall reliability of the signal value. Tab. 2 is a list of parameters that demonstrate the overall quality of microarray. According to the guidelines recommended by Affymetrix, the average background values should be comparable. The percentage of present calls should be similar for replicate samples, where extremely low values possibly indicate poor quality. Affymetrix suggests 3 as a safe threshold value for the 3’/5’ ratios, and recommends caution if that threshold is exceeded. From the information provided in Tab. 2, the average background values, percentage of present calls and the 3’/5’ ratios all indicate that our microarray data is of high quality, and is reliable for following analysis. In Fig. 3, scatter plots of MOFs to MFGEs and of MESFs to CMESFs are shown. Take the upper left scatter plot as an example. The abscissa value of each spot denotes the signaling intensity of CMESFs, and ordinate value indicates the signaling intensity of MFGEs. Scatter plots in Fig. 3 tell us that the expression profiles of MOFs, MESFs, MOFs and CMESFs are similar to each other, while MFGEs are outliers of these five feeder cell lines.

2.3 Supporting-related genes

As described in Fig. 1, from the 52,865 probe sets of GeneChip® Rhesus Macaque Genome Array, we filtered the target probe sets by the following criteria: first, the probe set must call present in the supportive feeder cell lines; second, the expression level in supportive feeder cell lines has to be two times more than that in non-supportive feeder cell line (MFGE). After filtration, 848 raw candidates were obtained. We were only interested in the gene products that were located in the extracellular region or on the membrane of fibroblast cells. After deleting the probe sets which aim at the same genes and searching against the PubMed, we

Tab. 1 A_{260} to A_{280} ratio and concentration of RNAs from 5 feeder cell lines

Cell Line	A_{260}	$A_{260/280}$	$A_{260/230}$	Concentration(μg/μL)
MOFs	3.896	2.00	2.36	1.559
MESFs	4.448	2.00	2.36	1.779
CMESFs	3.439	1.99	2.31	1.376
MFGs	2.497	1.97	2.36	0.999
MFGEs	3.043	2.01	2.36	1.217

Tab. 2 Quality-determining parameters of different microarray data

	Average background	Percentage of present calls	Actin.3'/5'	Gapdh.3'/5'
CMESFs	33.85831	46.27447	1.2700875	1.116269
MESFs	33.11168	43.88915	1.4508982	1.325646
MFGs	35.28964	44.18613	1.4778908	1.124734
MFGEs	33.67706	47.17677	0.9679022	1.272379
MOFs	32.17206	44.28450	1.3148612	1.083990

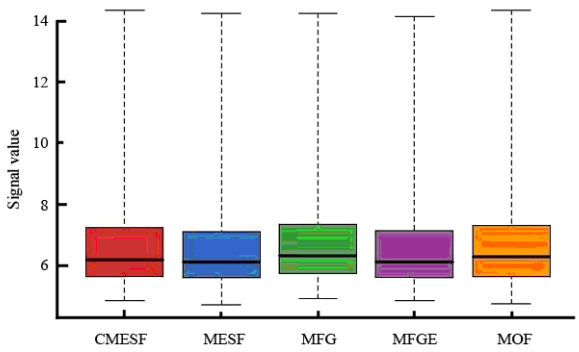

Fig. 2 Quality assessment of five microarray data

Signal value of five different feeder cell lines are evaluated in box plot.

finally got eight candidate genes as "supporting-relative genes", namely GREM2, bFGF, KITLG, DKK3, GREM1, AREG, SERPINF1 and LTBP1 (Tab. 3). Among them GREM1 and GREM2 are involved in BMP signaling pathway; DKK3 is involved in WNT signaling pathway; LTBP1 is related to TGF-β signaling pathway.

2.4 Confirmation of the microarray date by qPCR

In order to confirm the microarray data, we performed qPCR to detect the expression of three randomly chosen genes, SCF, bFGF and GREM2, in the eight "supporting-relative genes". Fig. 4 shows the qPCR result from SCF. qPCR results of bFGF and GREM2 are in accordance with their microarray data, but not shown here. The result ensures the reliability of our microarray data.

3 Discussion

Large and efficient expansion is a precondition for the further study and application of rESCs. Establishment and culture of all rESC lines to date required a layer of so-called feeder cells from MEFs that supply components necessary to sustain the self-renewal of rESCs. However, the disadvantages in using MEFs are due to their limited proliferating abilities, inter-batch variability (Richards et al, 2002; Park et al, 2003), and the possible introductions of mouse viruses and/or foreign proteins (Odorico, Kaufman & Thomson, 2001). In our previous study, we established a new culture system to expand rESCs with rhesus monkey feeder cells. Our results showed that the rESCs could grow undifferentiated on the four monkeys feeders (MESFs, MOFs, MFGs, and CMESFs), even on high-passage feeders. In order to find out which factors are important for the maintenance of undifferentiated growth and pluripotency of rESCs, we applied the microarray to obtain the expression profiles of all five different feeder cell lines. From the expression profiles, we can conclude that GREM2, bFGF, KITLG, DKK3, GREM1, AREG, SERPINF1 and LTBP1 mRNA are upregulated in

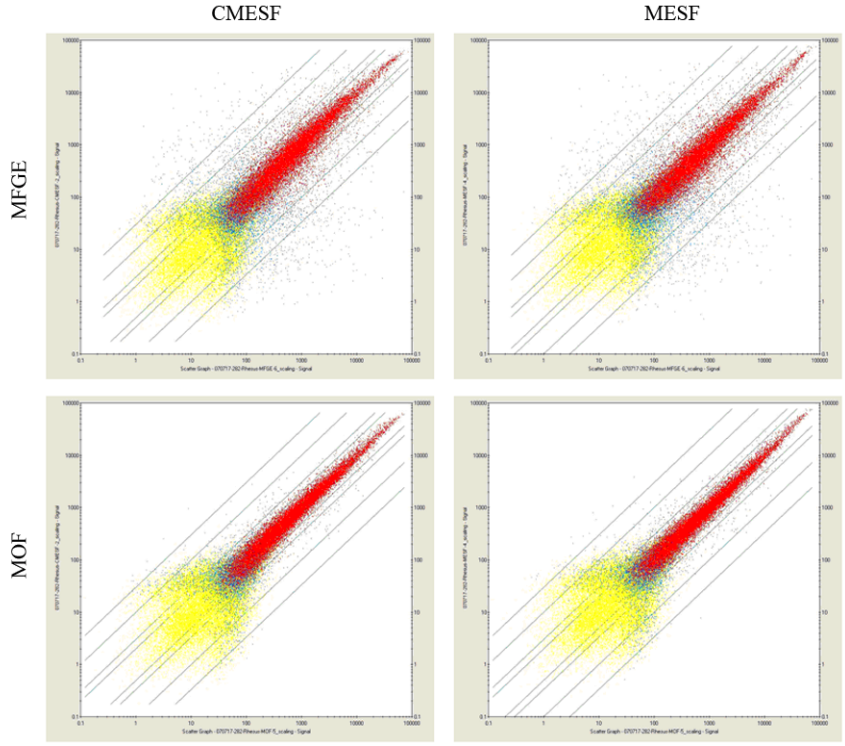

Fig. 3 Scatter plot of MFGEs vs CMESFs, MOFs vs CMESFs, MFGEs vs MESFs and MOFs vs MESFs

Tab. 3 Eight genes hypothesized to be related to self-renewal and pluripotency of rESCs after analyzing the microarray data

UniGene ID	Gene title	Gene symbol	Gene description	Reference
Hs.98206	Gremlin 2, cysteine knot superfamily, homolog (*Xenopus laevis*)	GREM2	BMP antagonist	Balemans & Van Hul, 2002
Hs.284244	Fibroblast growth factor 2 (basic)	bFGF	Basic fibroblast growth factor supports human embryonic stem cell self-renewal	Xu et al, 2005
Hs.1048	KIT ligand	KITLG	The survival of differentiating embryonic stem cells is dependent on the SCF-KIT pathway	Bashamboo et al, 2006
Hs.292156	dickkopf homolog 3 (*Xenopus laevis*)	DKK3	Wnt inhibitor	Niehrs, 2006
Hs.40098	Gremlin 1, cysteine knot superfamily, homolog (*Xenopus laevis*)	GREM1	BMP antagonist	Kueh et al, 2006
Hs.645475	amphiregulin (schwannoma-derived growth factor) /// similar to Amphiregulin precursor (AR) (Colorectum cell-derived growth factor) (CRDGF)	AREG	Amphiregulin is a member of the epidermal growth factor family; EGF stimulates proliferation of mouse embryonic stem cells	Heo, Lee & Han, 2006
Hs.645378	serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1	SERPINF1	Pigment epithelium-derived factor is a niche signal for neural stem cell renewal	Ramirez-Castillejo et al, 2006
Hs.49787	Latent transforming growth factor beta binding protein 1	LTBP1	The latent transforming growth factor-beta-binding protein-1 promotes *in vitro* differentiation of embryonic stem cells into endothelium	Gualandris et al, 2000

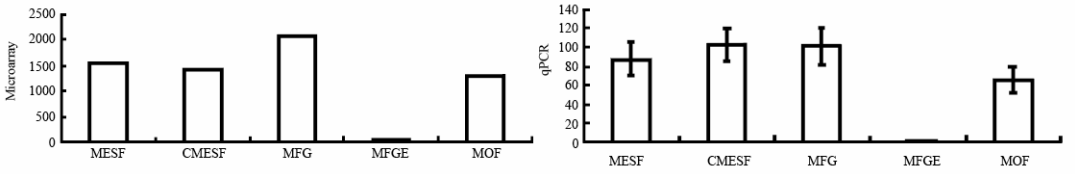

Fig. 4 Quantitative confirmation of the expression of SCF

Quantitative PCR results of bFGF and GREM2 also validate the microarray data (data not shown).

supportive feeder cell lines, but not in MFGE, which indicates many signaling pathways may be required to support the undifferentiated growth and maintenance of pluripotency in rESCs.

bFGF expression is 10 fold greater in MESFs, CMESFs, MFGs and MOFs than in MFGE, which implies that bFGF is an important cytokine for the undifferentiated growth of rESC. Many studies reveal that, unlike murine ESCs, rhesus ESCs are independent of LIF (Nakatsuji & Suemori, 2002) yet dependent on bFGF like human ESCs (Zhang et al, 2008). This is especially so in serum-free (Pei et al, 2003) or feeder-free (Zhang et al, 2006) culture systems. In Brandenberger's work, expression profiles of human (hESCs) implied that hESCs also produced bFGF, but not in sufficient amounts. So both hESCs and rESCs need exogenous bFGF from fibroblast feeder cells. bFGF in particular is an essential component for the maintenance of hESCs *in vitro* as withdrawal of bFGF results in ESCs differentiation and loss of TRA-1-60 and Oct3/4 (Kim et al, 2005). Reports suggest that FGF promotes self-renewal in ESCs by antagonising the BMP pathway through Smad1 inhibition (Nakayama et al, 2003; Aubin, Davy & Soriano, 2004), subsequently suppressing differentiation (Kim et al, 2005). Another branch of bFGF signaling is the PI3K/AKT signaling pathway. This is activated by FGF binding (Kannagi et al, 1983; Kim et al, 2005) and promotes self-renewal in ESCs through inhibition of MEK/ERK signaling, and/or up-regulation of ECM molecules, which are essential for maintaining ESC pluripotency *in vitro* (Kim et al, 2005).

Bone morphogenetic proteins (BMPs) are members of the TGFb superfamily and function via a different signaling pathway from that described for TGFb/activin/nodal signaling. The canonical BMP signaling pathway is initiated by the binding of BMP to

heterodimers of BMPR1a and BMPRII (Massague, 1996; Ying et al, 2003). This leads to activation of Smad1/5/8, which forms a heteromeric complex with Smad4 prior to translocation to the nucleus (Massague & Wotton, 2000; Besser, 2004; James et al, 2005). In contrast to mESCs, BMP signaling does not contribute to self-renewal in hESCs. In fact, it promotes rapid down-regulation of Nanog and Oct3/4 (Beattie et al, 2005) that subsequently induces trophoblast differentiation (Xu et al, 2002). The low levels of activated Smad1/5/8 found in undifferentiated hESCs increase during differentiation and may compete with active Smad2/3 to complex with co-Smad4. In this way, BMP signaling in hESCs may act as a regulatory element of TGFb/activin/nodal signaling (Besser, 2004).

In previous reports, when murine embryonic fibloblasts (MEFs) are used as feeder cells, bFGF induces the upregulation of Grem1 (Greber, Lehrach & Adjaye, 2007). As an antagonist of BMP (Sudo et al, 2004; Katoh & Katoh, 2006), Gremlin can work with high level of bFGF and maintain the self-renewal of human ESCs without the support from MEFs (Xu et al, 2005). Thus we may reach the conclusion that GREM1 and GREM2 are induced by bFGF, antagonize the expression of BMP proteins in rESCs, and function synergetically with bFGF to maintain the undifferentiated growth of rESCs.

The SCF-KIT pathway has been reported to play important roles in proliferation, differentiation and survival of a panel of progenitor cell types including haematopoietic stem cells (HSCs) (Engstrom, Karlsson & Jonsson, 2003; Young, Cambareri & Ashman, 2006), neuronal stem cells (Erlandsson, Larsson & Forsberg-Nilsson, 2004) and melanocyte precursors (Ito et al, 1999). It has been proposed that SCF-KIT pathway suppresses apoptosis to promote survival of these cell types. As for the roles of SCF-KIT pathway in ESCs, a correlation between Kit expression and pluripotency has been reported in murine ESCs (Palmqvist et al, 2005). Recently, Bashamboo et al (2006) found that Kit null ESCs died when induced to differentiate upon withdrawal of LIF in monolayer culture.

As a kind of secretory signaling molecules, TGF-β proteins have multiple functions. They were initially considered as growth factors regulating proliferation, differentiation, migration and apoptosis of cells. They are secreted as inactive precursors and then form large latent complexes with Latent transforming growth factor-beta binding proteins (LTBP) by disulfide bond (Munger et al, 1997). LTBPs are a family of high molecular weight matrix proteins. Four different LTBP (LTBP-1, -2, -3 and -4) genes are included in this family and have different affinities for TGF-β (Saharinen & Keski-Oja, 2000). By studying LTBP knockout mouse, Drews et al. proved that loss of LTBP1 decreased the activity of TGF-β (Drews et al, 2008). Therefore LTBP1 plays as a pivotal factor in maintaining undifferentiated state of ECSs by controlling the biological activity of TGF-β.

In summary, we have applied the microarray technique to find out what cytokine are differentially expressed in MFGE, compared with the supportive feeder cell lines. In order to confirm whether these cytokines are indeed paramount to support the rESCs in an undifferentiated state, further research should investigate the function of these soluble and cell-associated components with RNAi and over expression. Furthermore, we can finally determine the importance of the cytokines after we culture the rESCs without feeder cells or serum.

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