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Short communication

IDENTIFICATION OF MOLECULES DERIVED FROM HUMAN FIBROBLAST FEEDER CELLS THAT SUPPORT THE PROLIFERATION OF HUMAN EMBRYONIC STEM CELLS

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Abstract: The majority of human embryonic stem cell lines depend on a feeder cell layer for continuous growth *in vitro*, so that they can remain in an undifferentiated state. Limited knowledge is available concerning the molecular mechanisms that underlie the capacity of feeder cells to support both the proliferation and pluripotency of these cells. Importantly, feeder cells generally lose their capacity to support human embryonic stem cell proliferation *in vitro* following long-term culture. In this study, we performed large-scale gene expression profiles of human foreskin fibroblasts during early, intermediate and late passages using a custom DNA microarray platform (NeuroStem 2.0 Chip). The microarray data was validated using RT-PCR and virtual SAGE analysis.

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Abbreviations used: hESC – human embryonic stem cells; hFC – human fibroblast cells; hFF – human neonatal foreskin fibroblasts; HPC – hematopoietic progenitor cells; HSC – hematopoietic stem cells; MEF – mouse embryonic fibroblasts; MSC – mesenchymal stem cells; NSC – neural stem cells; PEDF – pigment epithelium-derived factor; SAGE – serial analysis of gene expression

Our comparative gene expression study identified a limited number of molecular targets potentially involved in the ability of human neonatal foreskin fibroblasts to serve as feeder cells for human embryonic stem cell cultures. Among these, the C-KIT, leptin and pigment epithelium-derived factor (PEDF) genes were the most interesting candidates.

Key words: Human embryonic stem cells, Feeder cells, DNA microarray

INTRODUCTION

Embryonic stem cells (ESC) were first derived from the inner cell mass of the murine blastocyst in 1981 [1]. Such cell lines were also later produced using human embryos [2]. Today, human ESC (hESC) are used for basic research to provide invaluable insight into the mechanisms underlying cell proliferation, differentiation, aging and regeneration. Current scientific efforts aim to move hESC-based technology from experimental research into the clinic. Most hESC lines are maintained on a feeder layer of separate origin. Mouse embryonic fibroblast (MEF) feeder cells were traditionally used to support the derivation and expansion of hESC lines. However, due to the risks, which include transmission zoonosis, a more clinical approach is required, so that the hESC are not exposed to xenogenic factors. Thus, feeder-free approaches have been developed. These include the supplementation of a high concentration of exogenous fibroblast growth factor 2 (FGF2); the addition of transforming growth factor β (TGFβ) and noggin proteins; the use of a matrix (such as Matrigel); or a combination of these approaches. Using defined media (e.g. mTeSR1 and mTeSR2 [3]) is also now widespread and allows for the effective expansion of hESC in feeder-free conditions. Despite these advances, the culturing of hESC on feeder cells of human origin, i.e. human fibroblast cells (hFC) [4], is still used for *in vitro* culturing and the expansion of some hESC lines (though feeder layers of mouse origin are used even more extensively). hFC have a number of advantages over mouse embryonic fibroblasts (MEF), both with regards to safety and convenience. Numerous hFC cell types have been used, but human neonatal foreskin fibroblasts (hFF) appear to be the most commonly used human feeder cell type worldwide.

The mechanisms underlying hESC-supporting feeder cell properties are currently not known in detail, but are suggested to be related to i) the secretion of factors into the culture media and/or ii) the expression of certain molecules over the cell surface membrane of hFC. Interestingly, fibroblasts (including MEF and hFF) can only support hESC proliferation *in vitro* during their early cell passages; spontaneous differentiation and cell death become evident in hESC plated on late passage hFC [5, 6]. Therefore, we hypothesized that comparing large-scale gene expression profiles of hFF during early, intermediate and late passages (i.e. the passages able and unable to support hESC proliferation *in vitro*) using a custom microarray platform [7] could identify factors underlying the ability of hFF to support the self-renewal of hESC. Once

identified, those factors could provide essential insight into the general biology of ESC and contribute to the establishment of more well-defined 'feeder-free' protocols for culture of hESC.

MATERIALS AND METHODS

Human neonatal foreskin fibroblast cells (hFF) were obtained from a commercial source (ATCC; cell line CCD-1112Sk) and expanded in hFF medium (IMDM, Invitrogen, USA) supplemented with 10% heat-inactivated FCS (Invitrogen) and 0.5% Penicillin/Streptomycin (Invitrogen) under regular culture conditions.
Human embryonic stem cells (hESC; SA002 line, Cellartis AB, Göteborg, Sweden) were co-cultured with mitotically inactivated (40 Gy) hFF in the presence of 4 ng/ml human recombinant FGF2 (Biosource International, USA) to assess the latter cells' ability to support hESC proliferation *in vitro* in an undifferentiated stage.
Cells were harvested for RNA isolation from hFF at sub-confluency during passages 6-15, 20, 25, 30 and 34, then purified following the RNeasy Kit protocol (Qiagen, USA), with DNase I treatment (Qiagen). RNA integrity was tested using both an ND-1000 spectrophotometer (NanoDrop, USA) and RNA Nano LabChip/2100 Bioanalyzer system (Agilent Technologies, USA). Total RNA for Sample I and Sample II was prepared by pooling equal quantities of individual total RNA samples from cells during passages 6-11 and 12-15, respectively. The fluorescent label incorporation was performed using a Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies, USA). Human Universal Reference RNA (Stratagene, USA) and dye-swap replicate amplification were used. The properties of the 'NeuroStem' custom microarray platform are described extensively in [7]. Briefly, the NeuroStem 2.0 Chip platform accounts for a total of 11532 individual oligonucleotides (69-71 nucleotides long), including 1312 oligonucleotides that match genes related to the growth and differentiation of stem cells, further supplemented with a large number of controls (in particular, for data normalization). All of the unique genes were spotted in quadruplicate over the same slide. Arrays were produced by the SweGene DNA Microarray Resource Centre, Department of Oncology at Lund University (Sweden) using a MicroGrid II 600R arrayer (Harvard BioRobotics, USA). For a detailed protocol of the platform manufacturing, application and analyses, see the Supplementary Materials and Methods at http://dx.doi.org/10.2478/s11658-010-0039-8. The microarray data validation included RT-PCR, SAGE cross-library analysis and ELISA (see the Supplementary Materials and Methods).

RESULTS

In concordance with the results of previous research [4, 5], we observed that a high rate of proliferation and a low grade of spontaneous differentiation in

hESC cultures could not be achieved with hFF older than passages 11-12. The hFF derived from later passages were unable to support hESC proliferation *in vitro*, and the rate of spontaneous differentiation increased gradually, finally approaching 100%, co-inciding with massive cell death (Suppl. Figs 1 and 2). Using laboratory records, we retrospectively analyzed the growth rates of hFF routinely used to support hESC in our laboratory (raw data from > 200 passages). We observed that the hFF appear morphologically unaltered (i.e. do not appear hypertrophic) and display an equal growth rate from passage 4 to 25. Morphological changes become evident in hFF only by passage 25 (Suppl. Figs 3 & 4). We therefore hypothesized that it is between passages 12 and 25 that hFF may undergo molecular changes which lead to the loss of their ability to support hESC proliferation.

To identify the molecular expression changes in aging fibroblasts, hFF were cultured for up to 35 passages, and RNA samples were purified and tested for integrity. The following hFF samples were selected for the microarray study using a custom oligonucleotide microarray platform (NeuroStem 2.0 Chip):

I. Passages 6-11 (i.e. hFF that are able to support hESC proliferation);
II. Passages 12-20 (i.e. hFF that are unable to support hESC proliferation, but are morphologically unaltered and have same growth rate as those in Passages 6-11);
III. Passage 25 (i.e. hFF that are morphologically altered but have the same growth rate as those in Passages 6-20); and
IV. Passage 30 (i.e. hFF that are severely morphologically altered, and have a lower growth rate than those in Passages 6-25).

Using a conservative set of criteria, we were able to identify a group of 232 gene targets (2.01% of the total number of genes screened) with an expression in Sample I that is significantly different to that in Samples II-IV. In the latter Samples, a significant group of genes were related to the cell cycle, most likely responsible for the replicative aging process occurring in the hFF, although not reflected in the growth rate at this stage. Six genes encoding secreted molecules and one gene controlling secretion were identified among the gene targets (Tab. 1), including pigment epithelium-derived factor (*PEDF*; serpin peptidase inhibitor, clade F member 1; α2 antiplasmin; also used to be known as *EPC1*) and leptin (*LEP*; Fig. 1). Moreover, there were 43 additional gene products with expressions different in Sample I vs. Samples II-IV that were related to the cell surface membrane (Suppl. Tab. 1, Fig. 2), including *C-KIT* (also known as *CD117* and mast/stem cell growth factor receptor (*SCFR*)).

In a subsequent analysis, we matched the target list to the GEO (Gene Expression Omnibus) database (NCBI), in which 3 entries related to feeder cells are available to the scientific community. The Serial Analysis of Gene Expression (SAGE) libraries of primary (passage 0) human foreskin cells (GSM1) and those of passages 15-18 (GSM14916), and the MEFs of passage 3

Tab. 1. Secreted factor-encoding genes with a different expression level in hFF after passage 11.

No.	Gene index	Sample Cy3 *vs.* reference Cy5				Sample Cy5 *vs.* reference Cy3			
		I	II	III	IV	I	II	III	IV
1.	***VTN***	-0.743	0.151	0.433	-0.979	-0.028	0.900	0.675	-0.103
2.	*PRSS23*	0.737	0.349	0.496	1.214	0.506	0.172	0.613	1.145
3.	*WNT2*	0.745	0.368	0.373	1.119	0.460	0.093	0.090	1.006
4.	*TNFRSF1A*	1.063	0.657	0.543	0.861	0.886	0.513	0.340	0.907
5.	***PEDF***	0.125	-0.606	-1.095	-1.324	0.476	-0.486	-0.687	-1.132
6.	***LEP***	1.515	0.441	ND	ND	1.492	0.786	-0.667	ND
*	***HOXD4***	-1.389	-0.328	0.156	-0.978	-1.472	-0.301	0.075	-0.981

I – human feeder cells from passages 6-11; II – from passages 12-20; III – from passage 25; IV – from passage 30. Values, mean Log 2 Ratio. Only samples with an expression that was different in the > 0.25 Log2 Ratio range at stage I/II and a consistent pattern of expression are shown. **Bold** highlights entries with a > 0.25 Log2 Ratio range at stage I/II in both Sample Cy3 vs. Reference Cy5 and Sample Cy5 vs. Reference Cy3 datasets. The entries are sorted based on the average value of the Log2 Ratio change of both datasets at stage I/II. ND, not detected. *Hoxd4 is not a secreted factor but is believed to affect the secretion process.

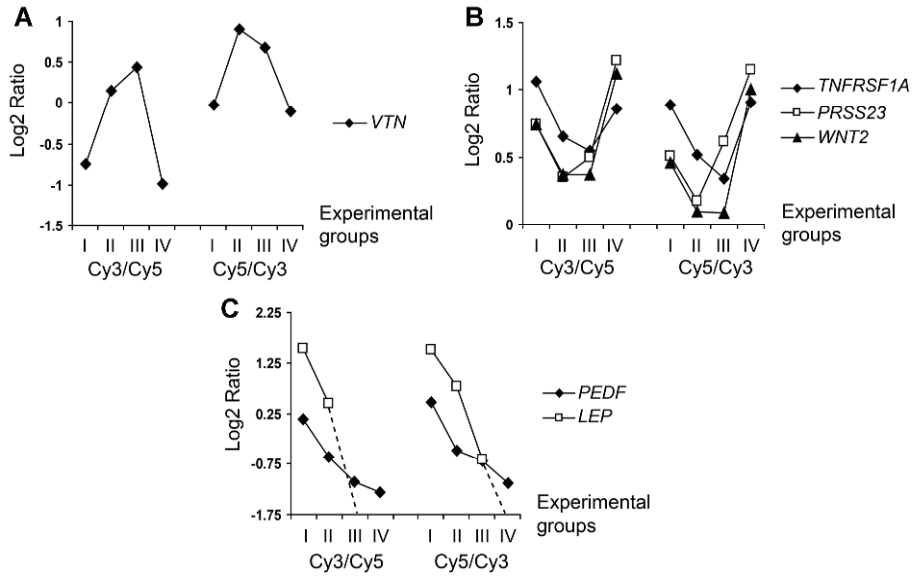

Fig. 1. Secreted factor-encoding genes with a different expression level in hFF after passage 11 (experimental groups, abscissa) plotted as a function of the Log2 Ratio (ordinate): bell-shape (A), u-shape (B) and down-regulation (C) patterns. Left panel: Sample Cy3 vs. Reference Cy5. Right panel: Sample Cy5 vs. Reference Cy3. I, passages 6-11; II, passages 12-20; III, passage 25; IV, passage 30. The dashed line in (C) indicates that gene expression of leptin (*LEP*) was below the detection limit. All of the samples shown have their expression altered to the > 0.25 range at stage I/II.

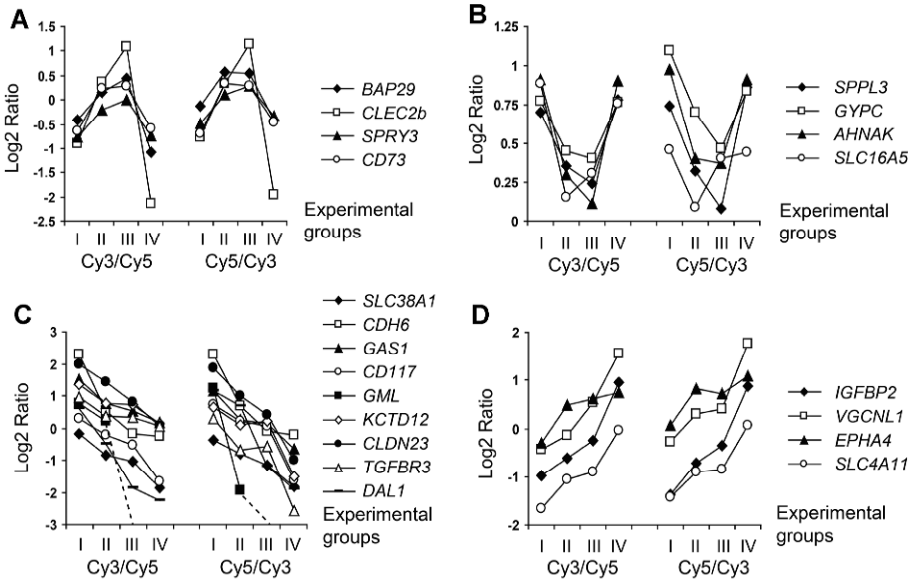

Fig. 2. Cell surface membrane-related genes with a different expression level in hFF after passage 11 (experimental groups, abscissa) plotted as a function of the Log2 Ratio (ordinate): bell-shape (A), u-shape (B), down-regulation (C) and up-regulation (D) patterns. Left panel: Sample Cy3 vs. Reference Cy5. Right panel: Sample Cy5 vs. Reference Cy3. I, passages 6-11; II, passages 12-20; III, passage 25; IV, passage 30. The dashed line in (C) indicates that the gene expression of *GML* was below the detection limit. All of the samples shown have their expression altered to the > 0.5 range at stage I/II for (A, C) and > 0.25 for (B, D).

(GSM7759; [8]) were downloaded, and the SAGE tags for the corresponding gene targets were identified using SAGE Map software (NIH). Targets identified using the NeuroStem Chip were matched to those libraries. Importantly, a large proportion of gene targets identified using microarray technology (including secreted, secretion-related and cell surface membrane-related ones) were also identified in the SAGE libraries: 142 redundant tags for *Homo sapiens*, matching 42 unique genes, and 137 redundant tags for *Mus musculus*, matching 45 unique genes, were identified for 50 target genes tested. Of those tags, 42 (matching 33 unique genes) were expressed in the human GSM1 and GSM14916 SAGE libraries, and 29 (matching 24 unique genes) were expressed in the mouse GSM7759 SAGE library. Suppl. Tab. 2 lists 37 unique genes encoding secreted, secretion-related and cell surface membrane-related products identified in one of the three SAGE libraries analyzed.

The normalized abundance of the individual tags was also compared between human feeder cell-derived SAGE libraries approximately corresponding to Sample I and Sample II of our experiment. The resulting trends were compared to that derived from microarray analysis (Suppl. Fig. 5). Despite the principally different manners in which the data was generated, in a number of cases, we

were able to observe high levels of similarity between the expression patterns observed in our microarray experiments (Tab. 1, Suppl. Tab. 1) and in human SAGE feeder cell-derived libraries.

To further validate the gene expression changes observed in the microarray experiments, we performed RT-PCR analysis. Herein, the expression of a number of selected target genes was investigated in a wide range of hFF-derived samples (passages 6-15, 20, 25, 30, 34; thus exceeding the assay of raw samples assessed in the microarray experiments). In particular, in concordance with microarray data, RT-PCR demonstrated that the expression of *C-KIT* and *LEP* rapidly dropped with hFF passage number. A wider range of hFF samples allowed us to identify with higher precision that the expression of *C-KIT* and *LEP* decreased after passage 9 and nearly completely disappeared after passage 25 (Suppl. Fig. 6). Furthermore, using ELISA, we confirmed that the levels of secreted PEDF declined over increasing passage number. The linear regression curve obtained for the PEDF antigen had an R2 value of 0.943. The normalized PEDF values were 16 ng/ml, 12.16 ng/ml and 11.52 ng/ml for passage numbers 9, 16, and 21, respectively.

DISCUSSION

It is generally recognized that feeder cells (including MEF and hFF) are only able to support the proliferation of hESC in an undifferentiated condition for a limited number of passages, from 11-12 to 15 for hFF [3, 4] and from 3-4 to 6-7 for MEF [9]. However, little is known about the basic mechanisms underlying the hESC-supportive features of either type of feeder cell. In this study, we aimed to identify genes that may encode molecules supportive for the proliferation and pluripotency of hESC. Using a custom microarray platform, we identified a limited number of gene targets (232) with expressions that were significantly different between hFF passages termed early (passage numbers ≤ 11) and late (passage numbers > 11). We obtained support for our microarray-generated data using RT-PCR studies and virtual SAGE, based upon the analysis of expression of selected genes in an array of publicly available SAGE libraries. Notably, a degree of overlap between the available human libraries and across species (including those for secretion- and cell surface membrane-related genes) supports the notion that the mechanisms underlying the ability of human and murine feeder cells to support hESC proliferation might be identical.

As expected, a significant number of the gene targets identified in our study were related to the cell cycle, in concordance with the aging process in hFF. Similarly, a limited number of identified gene targets (50 genes) were related either to cell secretion (i.e. encoding secreted molecules or controlling secretion), or to the cell surface membrane (Tab. 1 and Suppl. Tab. 1). Several studies suggest that the hESC-supportive features of fibroblasts are primarily (or even entirely) based on secreted factors [10, 11]. Therefore, we investigated the group of genes encoding the secreted proteins. Among the latter, we identified

vitronectin (*VTN*), serine protease 23 (*PRSS23*), wingless-type MMTV integration site family member 2 (*WNT2*), tumor necrosis factor receptor superfamily member 1A (*TNFRSF1A*), pigment epithelium-derived factor (*PEDF)* and leptin (*LEP*). Additionally, the homeobox D4 (*HOXD4*) gene, believed to affect the secretion process, was also identified. Importantly, only two of those genes, *PEDF* and *LEP*, were consistently down-regulated throughout replicative senescence (Fig. 1).

It is now recognized that leptin inhibits apoptosis and enhances cell proliferation via specific activation of certain signalling pathways. Leptin and the leptin receptor are expressed in fibroblasts, and it has been observed that leptin receptors are expressed in various stem cell types, including human mesenchymal stem cells (hMSC), haematopoietic stem and progenitor cells (hHSC, hHPC), and hESC [7]. Similarly, PEDF function has been linked to the maintenance of stem cell populations, namely that of neural stem cells (NSCs) [12]. PEDF is also considered to be involved in the maintenance of adult 'niches' of NSCs and to promote their proliferation via the activation of signalling pathways, while a blockade of endogenous PEDF decreases the proliferation [12]. Most interestingly, a few independent studies have linked alterations of PEDF expression in human fibroblast and fibroblast-like cells undergoing replicative senescence [13-15], which illustrates that senescent cells may not be able to produce PEDF transcripts. Previous studies have identified PEDF in a mouse and human fibroblast cell-conditioned medium, suggesting that this protein could also be a potential regulator of hESC. Finally, in a more recent study, it was firmly established that PEDF supports the self-renewal of pluripotent hESC, promoting long-term growth of pluripotent hESC *in vitro* without further bFGF or TGFβ/Activin/Nodal ligand supplementation [16].

To fulfill their role in supporting stem cells, the secreted factors PEDF and leptin could utilize partial synergistic mechanisms, since both are recognized activators of the mitogen-activated protein kinase (MAPK) signalling cascade. This cascade potentially involves yet another factor identified in our study, namely C-KIT. This factor represents the sole entry in the cell surface membrane-related molecule group, which is linked not only to germ cell precursors and stem cell subtypes, but also to the MAPK signalling pathway and to leptin itself [17, 18]. We hypothesize that the PEDF, leptin and C-KIT-mediated systems may play important roles in controlling the hESC-supportive features of hFF, while other molecular mechanisms may also be involved. Among these is the well-documented FGF2 pathway, while others remain to be identified. We strongly believe that performing a similar analysis with various types of fibroblast cells (including MEF and human fibroblasts of various biological origin) and follow-up functional analyses of the identified molecular targets (as applied to the assay of hESC cell lines), could help to identify these. Using available feeder-free cell expansion systems (including mTeSR1 and mTeSR2 media) could be most useful in this context. This would be indeed most useful for the further development of feeder-free/xeno-free methods of hESC expansion.

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