Comparative Expression Profiles of mRNAs and microRNAs Among Human Mesenchymal Stem Cells Derived From Breast, Face, and Abdominal Adipose Tissues

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We determined the expression of both mRNAs and microRNAs (miRNAs) from human mesenchymal stem cells BM19, FM30, and AM3, which is derived from breast, face, and abdominal adipose tissues, respectively. BM19, FM30, and AM3 cells exhibited considerably similar mRNA profiles, and their 1,038 abundantly common genes were involved in regulating six cell adhesion and three cytoskeleton remodeling processes among the top ten GeneGo canonical pathway maps. The 39 most abundant miRNAs in AM3 cells were expressed at very similar levels in BM19 cells. However, seven abundant miRNAs (miR-19b, miR-320, miR-186, miR-199a, miR-339, miR-99a, and miR-152) in AM3 cells were expressed at much lower levels than that in FM30 cells, and 38 genes targeted by these miRNAs were consequently upregulated more than 3-fold in FM30 cells compared with AM3 cells. Therefore, autologous abdominal adipose-derived mesenchymal stem cells are suitable for tissue engineering of breast reconstruction because of very similar expression profiles of mRNAs and miRNAs between AM3 and BM19 cells. Conversely, abdominal AM3 cells might not be suitable for facial rejuvenation, since the 38 highly expressed genes targeted by miRNAs in FM30 cells might play an important role(s) in the development of facial tissue.

Key Words: adipose, genes, mesenchymal stem cells, microRNAs
(*Kaohsiung J Med Sci* 2010;26:113–22)

Mesenchymal stem cells (MSCs) have been shown to have the ability to differentiate into multiple mesodermal lineages such as adipocytes, osteoblasts, and chondrocytes [1,2]. Thus MSCs are potentially very useful for tissue engineering and regenerative medicine [3–5].

Received: Aug 30, 2009 Accepted: Oct 20, 2009
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Human MSCs have been isolated from several tissue sources, including adipose tissues [6–10]. Human adult adipose tissues are highly abundant and relatively easy to procure with low risk. In our laboratory, we developed a new culture method that greatly accelerates the growth rate and prolongs the life span of human adipose-derived MSCs (hAD-MSCs) using a growth medium with low calcium and supplemented with the antioxidants N-acetyl-L-cysteine and L-ascorbic acid [6]. Furthermore, hAD-MSCs isolated using this newly developed method from abdominal subcutaneous adipose tissue were used successfully

to regenerate *in vivo* new adipose tissue of predefined shape and three-dimensions on scaffolding made with three commonly used biomaterials [11].

Genome-wide mRNA expression profiling has recently been used to identify the core features of several MSCs and the signature genes of each group of MSCs derived from different origins [12–14]. MicroRNAs (miRNAs) that are single-stranded non-coding RNAs of approximately 22 nucleotides have been identified in various organisms, including mammals. Mammalian genomes encode many hundreds of miRNAs, which are predicted to regulate negatively expression of as many as 30% of protein-coding genes [15–17]. Although the biological functions of most miRNAs are unclear, some miRNAs appear to participate in controlling cell proliferation, differentiation, and apoptosis in animals [18,19]. Thus miRNAs may play key roles in self-renewal and differentiation of MSCs.

Tissue engineering using hAD-MSCs derived from abdominal adipose tissue may be a promising method to generate new tissues for tissue restorations such as breast reconstruction and facial rejuvenation. Therefore, it important to compare the molecular characteristics of hAD-MSCs derived from breast, face, and abdominal adipose tissues. In this investigation, the expression profiles of both mRNAs and miRNAs from the same RNA samples of hAD-MSCs newly isolated from breast and face adipose tissues (designated as BM19 and FM30) were compared with those of previously reported hAD-MSC3 derived from abdominal adipose tissue (designated as AM3) [20] to understand the genetic bases for their similarities and differences.

Methods

Cell culture

Human hAD-MSCs BM19 and FM30 were newly isolated from breast and face adipose tissues, respectively, as previously described [8,11,20]. The patients gave informed consent and Institutional Review Board approval was obtained by Kaohsiung Medical University Hospital. These hAD-MSC cells were cultured in K-NAC medium that is a modified MCDB 153 medium (Keratinocyte-SFM; GIBCO-Invitrogen Corp., Carlsbad, CA, USA) supplemented with 2 mM N-acetyl-L-cysteine (A8199; Sigma, St. Louis, MO, USA) and 0.2 mM L-ascorbic acid 2-phosphate (A8960; Sigma).

Profiling of mRNAs

Total RNAs from BM19 and FM30 cells were extracted using TRIZOL reagent (Invitrogen), and the same total RNAs from each sample were used for both mRNA microarray analysis and miRNA quantitation. The mRNA profiling of duplicate samples was analyzed using Affymetrix Human Genome U133 plus 2.0 GeneChip® according to the manufacturer's protocols (Santa Clara, CA, USA) and the Microarray Core Facility of National Research Program for Genomic Medicine of the National Science Council in Taiwan. This Affymetrix GeneChip® contains 54,675 probe sets to analyze the expression level of 47,400 transcripts and variants, including 38,500 well-characterized human genes. GeneChips from the hybridization experiments were read by the Affymetrix GeneChip® scanner 3000. The original data were processed using the GC-RMA algorithm and GeneSpring GX software version 7.3.1 (Silicon Genetics, Redwood City, CA, USA). The Affymetrix GeneChip® expression analysis can be used as a stand-alone quantitative comparison as the correlation between Affymetrix GeneChip® results and TaqMan real-time polymerase chain reaction (PCR) results show good linearity of $R^2 = 0.95$ by the MicroArray Quality Control Study—a collaborative effort of 137 scientists led by the US Food and Drug Administration [21,22]. The mRNAs of BM19, FM30, and AM3 [8,11,20] cells were also analyzed for network and signaling pathways using MetaCore analytical suite (GeneGo Inc., St Joseph, MI, USA). The MetaCore includes a curated database of human protein interaction and metabolism, and thus it is useful for analyzing a cluster of genes in the context of regulatory network and signaling pathways.

Profiling of miRNAs

The expression levels of 250 human miRNAs were determined using the TaqMan MicroRNA Assay (Applied Biosystems, Foster City, CA, USA) as described previously [20,23–25]. In brief, TaqMan MicroRNA Assays include two steps: stem loop reverse transcription (RT) followed by real-time quantitative PCR. (90 ng/Rx with 24-multiplex primers) Each 10 uL RT reaction that includes 90 ng total RNA, 50 nM stem-loop RT primers, 1 × RT buffer, 1.25 mM each of dNTPs, 0.25 U/uL RNase inhibitor, and 10 U/uL MultiScribe Reverse Transcriptase was incubated in a PTC-225 Peltier Thermal Cycler (MJ Research, Watertown, MA, USA) for 30 minutes each at 16°C and at 42°C, followed by

5 minutes at 85°C, and then maintained at 4°C. RT products were diluted 20 times with dH2O prior to setting up the PCR reaction. Real-time PCR for each miRNA was carried out in triplicate, and each 10 uL reaction mixture included 2 uL of diluted RT product, 5 uL of 2 × TaqMan Universal PCR Master Mix and 0.2 uM TaqMan probe. The reaction was incubated in an Applied Biosystems 7900HT Sequence Detection System at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The threshold cycle (Ct) is defined as the fraction cycle number at which the fluorescence exceeds the fixed threshold of 0.2. Total RNA input was normalized based on the Ct values of the TaqMan U6 snRNA assay as an endogenous control. The fold change was calculated as $2^{-\Delta Ct} \times K$, where $\Delta Ct = [Ct_{miRNA} - Ct\ U6_{snRNA}]$ and K is a constant.

Target identification of miRNAs

The potential target genes of miRNAs were predicted using the TargetCombo open source software [26] which predicts targets by the union of miRanda [27], PicTar [28], and TargetScanS [29] with a cutoff *p* value < 0.05 [30]. The impact of miRNAs on protein output recently showed that although some targets are repressed without detectable changes in mRNA levels, those translationally repressed by more than a third also display detectable mRNA destabilization, and for the more highly repressed targets, mRNA destabilization usually comprises the major component of repression [17]. Therefore, comparative profiling of miRNAs and mRNAs from the same samples of different cell types may identify the putative targets of miRNAs [20,25]. The expression levels of the predicted target mRNAs were analyzed by the Volcano plot using a parametric test and Benjamini-Hochberg false discovery rate for multiple testing corrections. The differentially expressed mRNAs were defined by fold-changes of more than three and a *p* value cutoff of 0.05. Thus the miRNA targets were identified by inverse relationships between expression levels of miRNAs and their target mRNAs in AM3 and FM30 cells.

RESULTS

Expression profiling of mRNAs

The genome-wide mRNA expression profiles of human breast BM19 and face FM30 cells were determined using the Affymetrix human genome U133 plus 2.0 GeneChip. The mRNA expression of BM19 and FM30 cells was compared with that of abdominal AM3 cells [20] and a very similar pattern was found, with a Pearson correlation R^2 values of 0.938 and 0.920, respectively (Figures 1A and 1B).

When compared with AM3 cells, there were 79 genes in BM19 cells and 112 genes in FM30 cells, which are abundantly (> 20-fold of the overall mean) differentially (> 3-fold) expressed, found to be upregulated (Supplementary Tables S1 and S2). These genes included several chemokine ligands and interleukins. Conversely, there are 44 upregulated genes in AM3 when compared with BM19 cells and 54 upregulated genes in AM3, including matrix metallopeptidase 1, when compared with FM30 cell (Supplementary Tables S3 and S4).

Using MetaCore analytical suite, the 1,038 gene probes commonly expressed among AM3, BM19, and FM30 cells were found to be involved in regulating six cell adhesion processes and three cytoskeleton remodeling processes among the top ten GeneGo canonical pathway maps (Figure 2).

Expression profiling of miRNAs

The expression profiles of 250 human miRNAs in BM19 and FM30 cells were quantitated using TaqMan MicroRNA Assays as described previously [20,23–25], and the expression level of each miRNA was indicated as fold change over U6 snRNA. The mean expression levels of triplicate analyses for 250 miRNAs from BM19 and FM30 cells were compared with that of AM3 cells [20] as shown in scatter plots (Figures 1C and 1D). A very close correlation ($R^2 = 0.998$) was found between BM19 and AM3 cells, while a much lower correlation ($R^2 = 0.724$) was obtained between FM30 and AM3 cells. The mean expression levels of 250 miRNAs from BM19, FM30, and AM3 cells [20] are given in Supplementary Table S5. We found that AM3, BM19, and FM30 cells expressed extremely low levels of the embryonic stem cell- and tissue- (liver, heart, muscle, pancreas, placenta and testis) specific miRNAs. The levels of the 39 most abundantly (> 20-fold U6 snRNA) expressed miRNAs in AM3 cells were compared with the corresponding values in BM19 and FM30 cells (Table 1). The 39 most abundantly expressed miRNAs in AM3 cells were found to be expressed at very similar levels (< 6-fold difference for only miR-199a, miR146b, and let-7i) in BM19

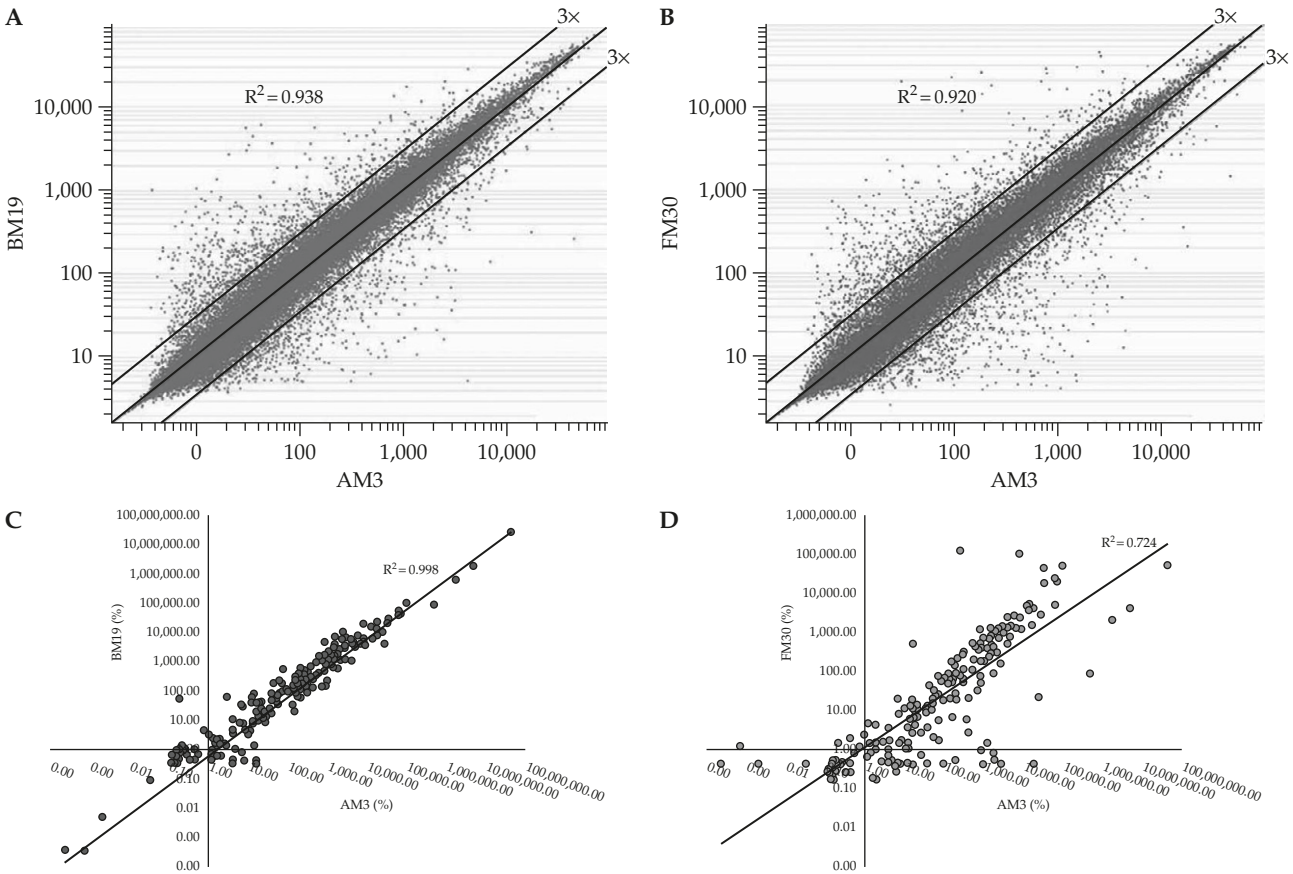

Figure 1. *Scatter plots and correlation analyses of mRNAs and microRNAs among AM3, BM19, and FM30 cells. The average mRNA expression levels of duplicate samples from (A) BM19 and AM3 cells and (B) FM30 and AM3 cells. The expression levels of more or less than three-fold are indicated by lines of 3×. The Pearson correlation of mRNA expression levels between AM3 and BM19 cells was found to be $R^2 = 0.938$, and that of AM3 and FM30 cells was $R^2 = 0.920$. The microRNA expression levels from (C) BM19 and AM3 cells; and (D) FM30 and AM3 cells. The Pearson correlation of microRNA expression levels between AM3 and BM19 cells was found to be $R^2 = 0.998$, and that of AM3 and FM30 cells was $R^2 = 0.724$.*

cells. However, seven extremely abundantly expressed miRNAs (miR-19b, miR-320, miR-186, miR-199a, miR-339, miR-99a, and miR-152) in AM3 cells were expressed at much lower levels (down-regulated more than 800-fold) than that in FM30 cells, while miR-222 and miR-146a were up-regulated more than 400-fold and 10-fold, respectively, in FM30 cells compared with AM3 cells.

Target identification of miRNAs

The targets of seven highly downregulated miRNAs (miR-19b, miR-320, miR-186, miR-199a, miR-339, miR-99a, and miR-152) in FM30 cells compared with AM3 cells were identified by inverse relationships between expression levels of miRNAs and their target mRNAs in AM3 and FM30 cells (Table 2). Thirty-eight target genes were found to be upregulated more than three-fold by the six downregulated miRNAs (except miR-99a) in FM30 cells compared with AM3 cells. Of these 38 genes, 11 genes (EREG, PAPPA, STC1, DLX1, PCDH9, LIF, PAX3, MYO16, TNFSF11, EYA4, and BEX1) were abundantly expressed in FM30 cells.

Discussion

In this study, we found that the expression profiles of mRNAs from breast BM19 and face FM30 cells were very similar to that of abdominal AM3 cells. The 1,038 abundantly expressed genes among AM3, BM19, and FM30 cells indicate that six of the top 10 network and signaling pathways are involved in cell adhesion processes. These results are in agreement with previous reports that the core signature transcriptomes of human MSCs isolated from bone marrow, cord blood,

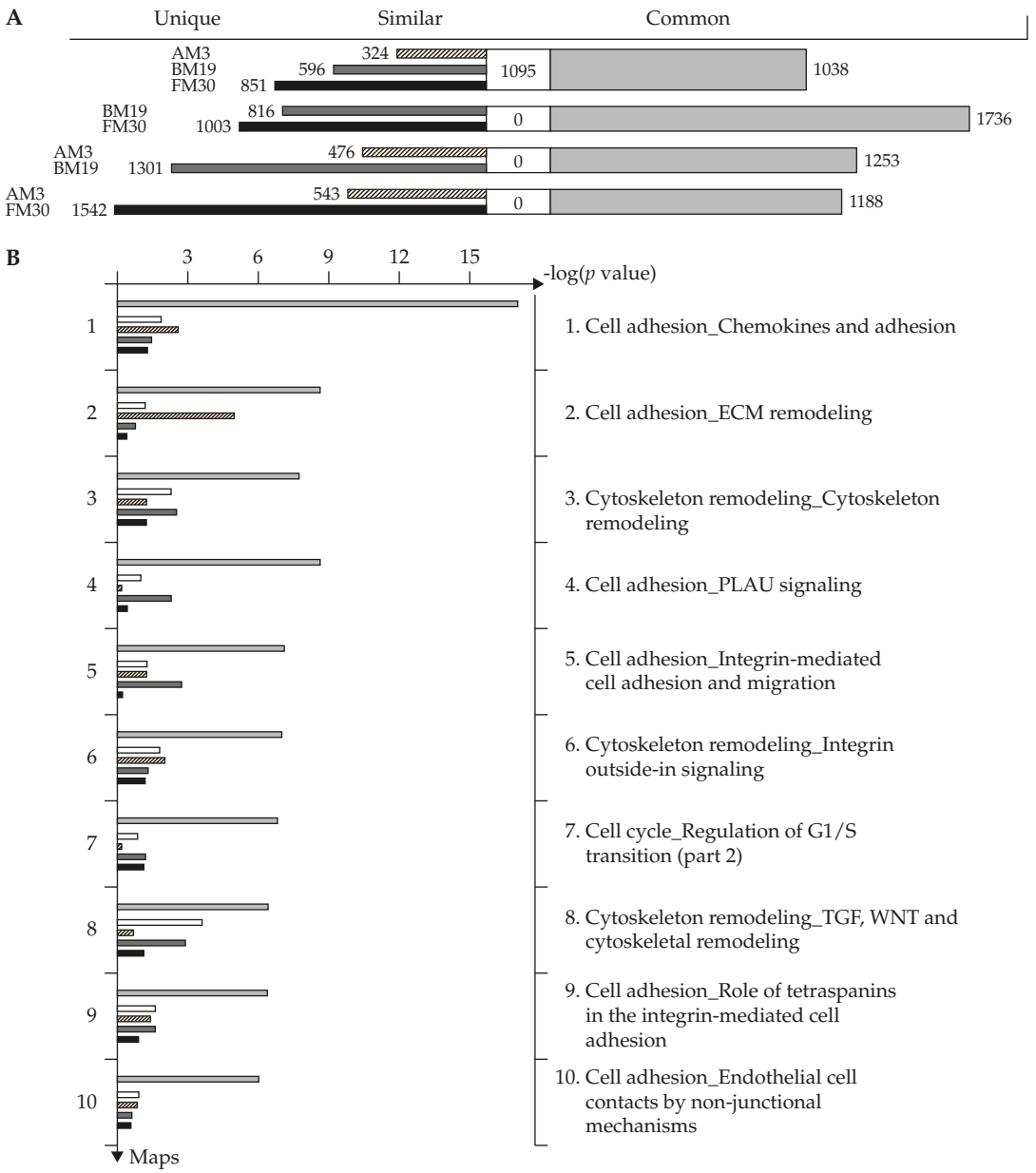

Figure 2. *Comparison of gene expression and GeneGo canonical pathway maps among AM3, BM19, and FM30 cells. (A) The parameters for comparison are set at a threshold of 3 with a p value of 0.05. The common genes are indicated by light grey bars. The "similar" genes that are common between any two of three cells are indicated by a white blank. The unique genes are marked (AM3, black/white strips; BM19, grey; and FM30, black). (B) The top 10 common GeneGo canonical pathway maps among AM3, BM19, and FM30 cells. The degree of "relevance" to different GeneGo ontology categories is defined by the p value, so that the lower random p value gets higher priority.*

amniotic fluid, and amniotic membranes include genes involved in the regulation of the extracellular matrix and adhesion [14,31].

Human miRNA changes during MSC differentiation have recently been studied, and 27 miRNAs were identified as being regulated during differentiation into adipocytes, osteocytes, or chondrocytes [32]. We found that the miRNA expression profile of BM19 cells was extremely similar to that of AM3 cells, but the miRNA expression profile of FM30 cells was less similar to that of AM3 cells. In FM30 cells, seven miRNAs (miR-19b, miR-320, miR-186, miR-199a, miR-339, miR-99a, and miR-152) were down-regulated more than 800-fold compared with AM3

Table 1. Expression levels of the 39 most abundantly expressed microRNAs in AM3 cells as well as the corresponding values in BM19 and FM30 cells

miRNAs	AM3	BM19	FM30	AM3/BM19	AM3/FM30
hsa-miR-19b	433406.50	259909.60	526.67	1.67	822.92
hsa-miR-320	48901.56	18751.85	41.80	2.61	1169.99
hsa-miR-186	17453.68	6067.10	21.32	2.88	818.76
hsa-miR-199a	4943.80	873.97	0.93	5.66	5313.38
hsa-miR-24	999.06	1024.00	526.02	0.98	1.90
hsa-miR-20a	695.43	431.97	202.30	1.61	3.44
hsa-miR-31	634.22	568.77	253.20	1.12	2.50
hsa-miR-16	632.81	384.14	51.59	1.65	12.27
hsa-miR-125b	341.94	296.07	182.28	1.15	1.88
hsa-miR-221	329.37	206.58	443.83	1.59	0.74
hsa-miR-146b	275.96	43.31	29.08	6.37	9.49
hsa-miR-339	246.21	102.82	0.23	2.39	1048.29
hsa-miR-99a	188.03	234.90	0.00	0.80	40251.62
hsa-miR-92	178.21	79.23	42.22	2.25	4.22
hsa-let-7b	165.44	138.24	15.72	1.20	10.52
hsa-miR-93	145.10	156.44	53.64	0.93	2.71
hsa-miR-125a	139.38	62.44	37.84	2.23	3.68
hsa-let-7a	125.68	157.57	50.43	0.80	2.49
hsa-miR-26a	89.85	37.29	12.77	2.41	7.04
hsa-miR-191	86.28	62.90	25.57	1.37	3.37
hsa-miR-21	82.87	48.70	23.57	1.70	3.52
hsa-miR-146a	79.64	202.15	1037.65	0.39	0.08
hsa-miR-27a	67.42	39.53	13.36	1.71	5.05
hsa-miR-29a	60.19	79.46	27.49	0.76	2.19
hsa-miR-214	49.86	32.97	15.37	1.51	3.25
hsa-miR-30c	46.60	43.65	8.26	1.07	5.64
hsa-miR-365	41.19	40.52	14.45	1.02	2.85
hsa-miR-19a	40.72	59.63	24.99	0.68	1.63
hsa-let-7i	40.06	11.05	5.26	3.63	7.61
hsa-miR-342	30.48	31.10	9.67	0.98	3.15
hsa-miR-140	28.72	69.70	14.54	0.41	1.98
hsa-miR-152	28.21	30.30	0.00	0.93	6038.74
hsa-miR-181d	28.15	34.31	12.84	0.82	2.19
hsa-let-7g	26.81	11.83	1.70	2.27	15.80
hsa-miR-376a	24.45	110.51	7.40	0.22	3.30
hsa-miR-127	23.75	59.67	12.69	0.40	1.87
hsa-miR-26b	21.36	11.21	3.20	1.91	6.67
hsa-miR-106b	20.11	26.73	11.42	0.75	1.76
					FM30/AM3
hsa-miR-222	2.54	5.26	1220.07		479.81
hsa-miR-146a	79.64	202.15	1037.65		13.03

cells. It is interesting that the abundant miRNAs miR-99a and miR-152 in AM3 cells were previously found to be almost absent in LM6 (LD-MSC6L) cells derived from lipoma adipose tissue, and that the highly upregulated genes HAS2, VNN1, COL11A1, and SLC16A6 targeted by miRNAs miR-99a and/or miR-152 may be responsible for a higher proliferation potential in LM6 cells compared with AM3 cells [10,20].

Thirty-eight genes targeted by these six miRNAs (except miR-99a) were upregulated more than three-fold in FM30 cells compared with AM3 cells, and the most abundant epiregulin gene was found to be expressed more than 1,400 times the overall mean in FM30 cells. These 38 highly expressed target genes might play an important role(s) in the development of facial tissue, and thus abdominal AM3 cells might not be suitable for tissue engineering of facial rejuvenation.

Table 2. Expression levels of 38 most abundantly differentially expressed genes targeted by microRNAs in AM3 and FM30 cells

Gene	miR-19b	miR-320	miR-186	miR-199a	miR-339	miR-152	FM30 mRNA	AM3 mRNA	FM30/AM3	Gene probes	UniGene	Description
EREG	19b	320					1426.00	402.90	3.54	205767_at	Hs.115263	Epiregulin
PAPPA			186		339		121.10	16.00	7.57	224940_s_at	Hs.694735	Pappalysin 1
STC1		320					97.88	6.62	14.79	204595_s_at	Hs.25590	Stanniocalcin 1
DLX1	19b		186				61.11	0.70	87.93	242138_at	Hs.407015	Distal-less homeobox 1
PCDH9		320					59.19	13.25	4.47	219737_s_at	Hs.654709	Protocadherin 9
LIF	19b						38.13	1.37	27.81	205266_at	Hs.2250	Leukemia inhibitory factor
PAX3				199a			31.36	0.81	38.53	231666_at	Hs.42146	Paired box gene 3
MYO16		320					27.12	1.91	14.24	215119_at	Hs.656587	Myosin XVI
TNFSF11		320					23.63	0.77	30.89	210643_at	Hs.333791	TNF (ligand) superfamily, member 11
EYA4		320					23.08	0.72	32.19	238877_at	Hs.596680	Eyes absent homolog 4
BEX1		320					22.67	0.03	680.78	218332_at	Hs.334370	Brain expressed, X-linked 1
MAB21L2	19b						19.22	0.59	32.52	210302_s_at	Hs.584852	Mab-21-like 2 (C. elegans)
GALNTL2			186	199a			17.71	3.29	5.39	236361_at	Hs.411308	UDP-N-acetyl-alpha-D-galactosamine
SMOC1	19b						9.47	1.96	4.83	222783_s_at	Hs.497349	SPARC related modular calcium binding 1
PDE4D				199a			8.32	2.11	3.94	204491_at	Hs.654358	Phosphodiesterase 4D, cAMP-specific
SLC25A37		320					7.73	1.97	3.92	226179_at	Hs.596025	Solute carrier family 25, member 37
HHIP	19b			199a			7.28	1.59	4.58	1556037_s_at	Hs.507991	Hedgehog interacting protein
TNFAIP3	19b						6.95	0.61	11.44	202644_s_at	Hs.591338	TNFα-induced protein 3
DUSP1						152	6.67	0.80	8.34	201041_s_at	Hs.171695	Dual specificity phosphatase 1
DKFZP564O0823	19b						6.59	0.84	7.85	225809_at	Hs.105460	DKFZP564O0823 protein
SEMA6D	19b	320					6.31	0.30	20.97	226492_at	Hs.511265	Sema domain, TM, and cytoplasmic domain
TRPC4		320					5.95	0.61	9.71	220817_at	Hs.262960	Transient receptor potential cation channel
DTNA	19b	320				152	5.68	0.91	6.25	227084_at	Hs.695993	Dystrobrevin, alpha
SAT1				199a			5.23	0.69	7.54	213988_s_at	Hs.28491	Spermidine/spermine N1-acetyltransferase 1
NPAS2	19b	320		199a			5.10	1.63	3.12	213462_at	Hs.156832	Neuronal PAS domain protein 2
CREB5		320					4.85	1.05	4.63	229228_at	Hs.437075	cAMP responsive element binding protein 5
FAM59A	19b						4.62	1.42	3.25	219377_at	Hs.444314	Family with sequence similarity 59, member A
DUSP4		320					4.21	0.59	7.16	204015_s_at	Hs.417962	Dual specificity phosphatase 4
NRK	19b						3.98	0.62	6.43	227971_at	Hs.209527	Nik related kinase
SLC1A1				199a			3.83	0.94	4.10	206396_at	Hs.444915	Solute carrier family 1
TMEM106B		320					3.80	1.14	3.32	222787_s_at	Hs.396358	Transmembrane protein 106B
TUBA4A	19b						3.74	0.82	4.54	212242_at	Hs.75318	Tubulin, alpha 4a
INHBA			186				3.73	1.22	3.06	204926_at	Hs.583348	Inhibin, beta A (activin A)
FOXP1	19b						3.41	1.09	3.13	235444_at	Hs.431498	Forkhead box P1
CLDN1			186				3.39	0.27	12.38	222549_at	Hs.439060	Claudin 1
NR4A2	19b	320	186				3.37	0.65	5.15	216248_s_at	Hs.563344	Nuclear receptor subfamily 4, group A2
ATP7A						152	3.24	0.99	3.27	205197_s_at	Hs.496414	ATPase, Cu++transporting, alpha
IVNS1ABP	19b						3.12	0.72	4.34	201362_at	Hs.497183	Influenza virus NS1A binding protein

Conversely, autologous abdominal AM3 cells are the most suitable for tissue restoration in breast reconstruction, since the expression profiles of mRNAs and miRNAs from abdominal AM3 and breast BM19 cells were found to be very similar.

Acknowledgments

We thank Dr Tsai-Ming Lin for providing human breast and face adipose tissues. We also appreciate the technical assistance by the research assistants at the Microarray Core Facility directed by Professor Pan-Chyr Yang of the National Research Program for Genomic Medicine of National Science Council in Taiwan. This investigation was supported in part by a National Genomic Medicine grant of the National Science Council in Taiwan (NSC95/96/97-3112-B-037-002), KMU Center of Excellence for Environmental Medicine Project (kmu-em-97-1.3c) to S.S.-L. Li, and the National Science Council, Taiwan (NSC 96-2314-B-037-002) to S.-D. Lin.

Supplementary Materials

Tables S1–S5 are available online at http://kjms.kmu.edu.tw.

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人類乳房，臉部及腹部組織來源的脂源性間葉幹細胞之基因與微核糖核酸表現分析

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本研究探討分別由乳房（**BM19**）、臉部（**FM30**）及腹部（**AM3**）組織來源的脂源性間葉幹細胞基因和微核糖核酸之表現。這三株細胞有相當類似的基因表現；利用 **GeneGo** 的分析軟體，其中有 **1,038** 個基因參與了六個與細胞黏附發展及三個與細胞骨架重塑相關的機轉。腹部（**AM3**）與乳房組織（**BM19**）而來的細胞株有 **39** 個主要的微核糖核酸表現量相似。然而，相對於腹部的 **AM3** 細胞，臉部的 **FM30** 細胞有 **7** 個表現量非常低的微核糖核酸（**miR-19b**，**miR-320**，**miR-186**，**miR-199a**，**miR-339**，**miR-99a** 和 **miR-152**）及 **38** 個微核糖核酸標的基因表現量大於腹部的 **AM3** 細胞 **3** 倍以上。因此，腹部的脂源性間葉幹細胞對乳房重建組織工程而言是一個較適合的來源，但不適合於臉部美容；此外，這 **38** 個微核糖核酸標的基因在臉部脂源性間葉幹細胞的高表現，或許在臉部組織的發展扮演著重要角色。

關鍵詞：脂肪，基因，間葉幹細胞，微核糖核酸

（高雄醫誌 **2010;26:113–22**）

收文日期：**98** 年 **8** 月 **31** 日

接受刊載：**98** 年 **10** 月 **20** 日

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