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Original Article

In vitro differentiation of bone marrow mesenchymal stem cells into endometrial epithelial cells in mouse: a proteomic analysis

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Abstract: Objective: Mouse bone marrow mesenchymal stem cells (BMSCs) have been demonstrated to differentiate into female endometrial epithelial cells (EECs) *in vivo*. Our previous studies demonstrated that BMSCs can differentiate in the direction of EECs when co-cultured with endometrial stromal cells *in vitro*. Here, we obtain and analyse differential proteins and their relevant pathways in the process of BMSCs differentiating into EECs by isobaric tags for relative and absolute quantitation (iTRAQ) proteomic analysis. Methods: A 0.4-μm pore size indirect co-culture system was established with female mice endometrial stromal cells (EStCs) restricted in the upper Transwell chamber and BMSCs in the lower well plate. After indirect co-culture for several days, the BMSCs were revealed to progressively differentiate towards EECs *in vitro*. Then, four groups were divided according to different co-culture days with single culture groups of BMSCs as controls. Proteins were detected using iTRAQ based on 2DLC-ESI-MS/MS and data were analysed by bioinformatics. Results: A total number of 311 proteins were detected, of which 210 proteins were identified with relative quantitation. Among them, 107 proteins were differentially expressed with a 1.2-fold change as the benchmark, with 61 up-regulated and 46 down-regulated proteins. Differential proteins CK19 and CK8 were epithelial markers and upregulated. Stromal marker vimentin were downregulated. Top canonical pathways was “remodeling of epithelial adhesions junctions” and “actin cytoskeleton signaling”. Top networks was “cell-to-cell signaling and interaction, tissue development and cellular movement” regulated by ERK/MAPK and α-catenin. Conclusion: To the best of our knowledge, this is the first preliminary study of differential protein expression in the differentiation process of BMSCs into EECs *in vitro*. We further elucidated BMSCs differentiated in the direction of EECs. In addition, ERK/MAPK and α-catenin played important roles by regulating core differential proteins in the “cell-to-cell signaling and interaction, tissue development and cellular movement” network.

Keywords: Bone marrow mesenchymal stem cells, endometrial epithelial cells; differentiation, proteomic analysis

Introduction

The human endometrium is a dynamic tissue that undergoes more than 400 cycles of regeneration, differentiation, and shedding during a woman’s reproductive years [1]. Endometriosis is the development of endometrial tissue outside of the uterus [2]. It affects 10%-15% of reproductive-age women and can cause pelvic pain and infertility [3]. Increasing evidence has shown that stem cells can contribute to endometrium and endometriosis [4-15]. Donor bone marrow mesenchymal stem cells (BMSCs) have been identified in the endometrium of female bone marrow transplant recipients; these cells appear to be histologically indistinguishable from the endogenous endometrial cells and express markers of glandular and stromal differentiation [8]. In addition, cells derived from the bone marrow of male donor mice have been found to contribute to EECs and stroma de novo in the eutopic and ectopic endometrium of female mice in vivo [4]. Stem cell markers oct-4 and c-kit were found to be expressed in ectopic epithelial cells, which suggests the stem cell origin [5].

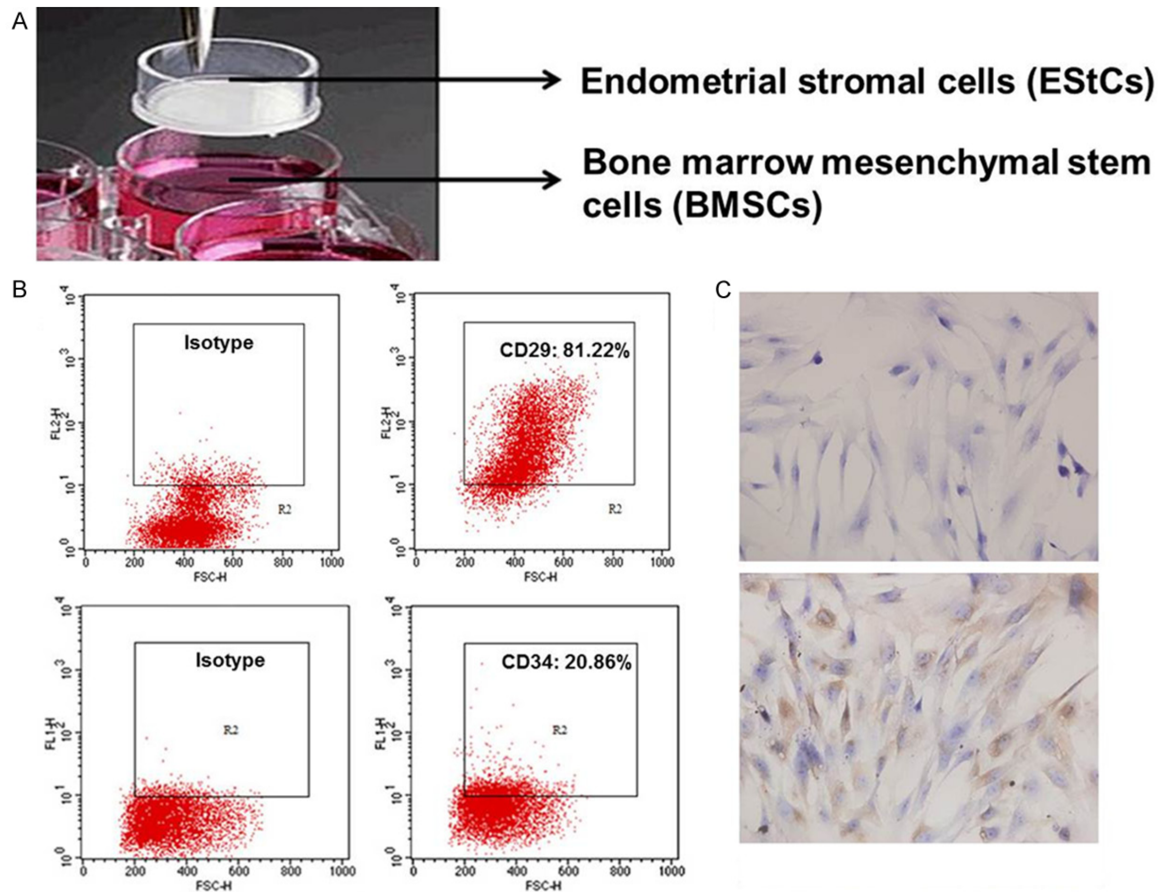

Figure 1. Isolation, characterization and co-culture of BMSCs and EStCs. A: Flow cytometric analysis of BMSCs cell surface markers expression from primary culture. Mesenchymal stem cell surface marker CD29 expression rate was 81.22% compared to the isotype and haemapoietic stem cell surface marker CD34 expression rate was 20.86% compared to the isotype. B: Primary culture of EStCs and immunocytochemistry characterisation. Negative control was stained with no primary antibody (up). EStCs were positive for stromal marker vimentin (down). Original magnifications × 400. C: Schematic diagram of co-culture system.

Recently, our group demonstrated in mice that BMSCs could differentiate in the direction of EECs in vitro by indirect co-culture BMSCs with endometrial stromal cells (EstCs) [9]. In this co-culture system, cells cannot pass the Transwell membrane (cross-culture room, pore size 0.4 μm, Millipore, USA), while small molecular substances can pass freely in the culture media. Thus, EStCs could induce BMSCs by paracrine signalling in the microenvironment, which is somewhat similar to environments that exist in vivo. After 7 days of indirect co-culture, expression of epithelial markers (cytokeratin 7, cytokeratin 18, cytokeratin 19, and epithelial membrane antigen) was tested in BMSCs using real-time RT-PCR, and expression of pan cytokeratin (CK) was tested using immunofluorescence staining. The mRNA levels of CK7, CK18 and CK19 were significantly higher in the co-culture system than in the control group (BMSCs cultured alone) ($P < 0.05$). Pan CK was positive in the co-cultured BMSCs and negative in the control group.

Isobaric tags for relative and absolute quantitation (iTRAQ), is a shot-gun-based technique that allows the concurrent identification and relative quantification of hundreds of proteins in up to 8 different biological samples in a single experiment [16, 17]. The technology has many advantages, such as having relatively high throughput due to sample multiplexing, and it has been shown to be suitable for the identification of low abundance proteins such as transcription factors [18].

To further illuminate BMSCs differentiate in the direction of EECs and obtain information in this

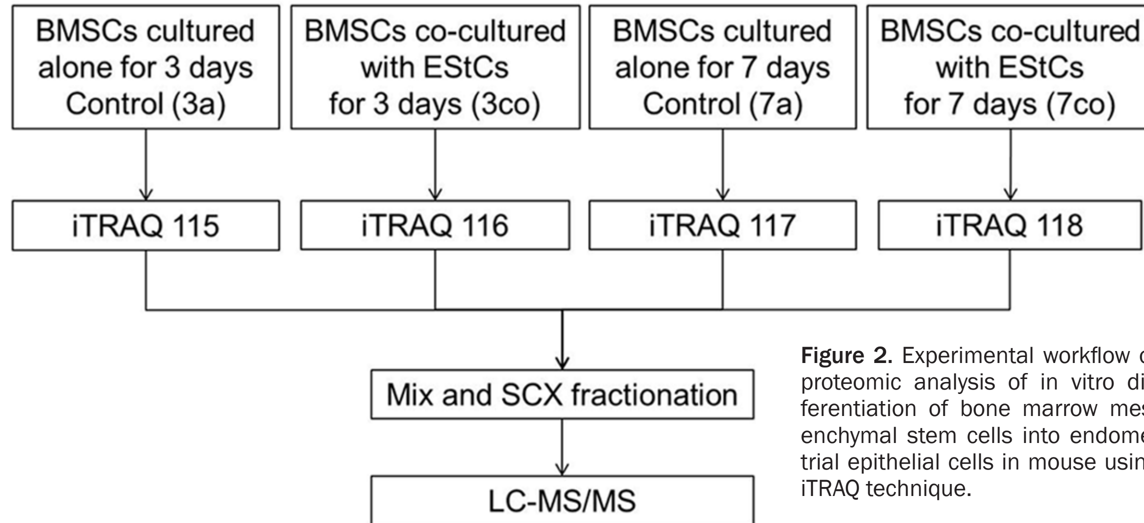

Figure 2. Experimental workflow of proteomic analysis of in vitro differentiation of bone marrow mesenchymal stem cells into endometrial epithelial cells in mouse using iTRAQ technique.

process, we tend to acquire differential proteins using iTRAQ and potential pathways by IPA (Ingenuity Pathway Analysis Software).

Materials and methods

Animals

All wild-type female C57BL/6 mice were purchased from Shanghai Slac Laboratory Animal Corporation. They were maintained under specific pathogen-free conditions with a light/dark cycle of 12/12 h. All experiments were approved by the institutional experimental animals review board of the Obstetrics and Gynaecology Hospital of Fudan University.

Indirect co-culture system of BMSCs and EStCs

The protocol for the isolation and flow cytometry characterization of BMSCs has been previously reported [9]. Isolation and immunocytochemistry characterization of EStCs was based on a previous report [19]. BMSCs and EStCs were co-cultured indirectly in transwell system (cross-culture room, pore size 0.4 μm, Millipore, USA). In brief, the BMSCs were seeded in the bottom of 6-well plate (6×10^5 cells/well) and the EStCs (6×10^4 cells/well) were seeded on the transwell membrane to separate the cells but allowing soluble factors to pass freely (**Figure 1A**). The culture medium was changed every 2 days. Totally, 2.9×10^7 BMSCs of passage 2 were seeded in 6-well plates co-culture system to generate enough protein of BMSCs co-cultured for 3 days and 7 days for proteomic analysis and Western blot.

Protein digestion, iTRAQ labelling and strong cation exchange fractionation (SCX)

All samples were centrifuged at 14,000 rpm for 60 s to collect cells in the Eppendorf microcentrifuge tubes. Each sample was then lysed by adding 50 μL of hypotonic buffer consisting of 7 M urea, 2 M thiourea, 65 mM DTT (DL-Dithiothreitol), 0.02% SDS cocktail and sonicated once. Total protein contents were determined using the commercial Bradford assay reagent (Bio-Rad, California, USA). A standard curve for the Bradford assay was made using γ-globulin as a control.

From each sample, 100 μg was denatured, and the cysteines were blocked as described in the iTRAQ protocol (Applied Biosystems, Foster City, CA). Each sample was digested with 0.2 mL of a 50 μg/mL trypsin (Promega, Wisconsin, USA) solution at 37°C overnight and labelled with the iTRAQ tags as follows (**Figure 2**): BMSCs cultured alone for 3 days, iTRAQ115; BMSCs co-cultured for 3 days, iTRAQ116; BMSCs cultured alone for 7 days, iTRAQ117 and BMSCs co-cultured for 7 days, iTRAQ118. The labelled samples were combined, desalted with Sep-Pak Vac C18 cartridge 1 cm^3/50 mg (Waters, USA), and fractionated using a Shimazu UFLC system (Shimazu, Japan) connected to a strong cation exchange (SCX) column (polysulfethyl column, 2.1 mm × 100 mm, 5 μm, 200 Å, The Nest Group, Inc. USA). SCX separation was performed using a linear binary gradient of 0-45% buffer B (350 mM KCl, 10 mM KH_2PO_4 in 25% ACN, pH 2.6) in buffer A (10

Table 1. One hundred and seven differential proteins (61 up-regulated and 46 down-regulated) were listed based on the ratio of BMSCs co-cultured for 7 days (7co) to those co-cultured for 3 days (3co)

No.	Gene symbol	Accession	Protein name	%Cov	118:116
1	Nes	IPI00453692.4	Isoform 1 of Nestin	29.67	0.05
2	Snrpb	IPI00114052.1	Small nuclear ribonucleoprotein-associated protein B	58.87	0.39
3	Sfrs7	IPI00222763.1	Isoform 1 of Splicing factor, arginine/serine-rich 7	73.03	0.50
4	Hist3h2a	IPI00221463.3	Histone H2A type 3	83.08	0.51
5	Hnrnpab	IPI00117288.3	Heterogeneous nuclear ribonucleoprotein A/B	37.54	0.52
6	Capza1	IPI00653841.1	Capping protein (actin filament) muscle Z-line, alpha 1	41.38	0.52
7	Rplp0	IPI00314950.2	60S acidic ribosomal protein P0	19.56	0.56
8	Sfpq	IPI00129430.1	Splicing factor, proline- and glutamine-rich	42.92	0.58
9	Tubb5	IPI00117352.1	Tubulin beta-5 chain	51.13	0.61
10	Psmb1	IPI00113845.1	Proteasome subunit beta type-1 precursor	53.75	0.63
11	Ppia	IPI00554989.3	Peptidyl-prolyl cis-trans isomerase	78.44	0.69
12	P4hb	IPI00133522.1	Protein disulfide-isomerase precursor	52.06	0.70
13	EG666548	IPI00752639.1	Similar to ribosomal protein L23a	32.90	0.70
14	Dync1h1	IPI00119876.1	Cytoplasmic dynein 1 heavy chain 1	23.13	0.72
15	mCG_17237	IPI00673288.1	predicted pseudogene 10116	71.04	0.72
16	Ywhab	IPI00760000.1	Isoform Short of 14-3-3 protein beta/alpha	49.18	0.72
17	Hmgn2	IPI00650026.1	High mobility group nucleosomal binding domain 2	53.13	0.73
18	Col1a1	IPI00329872.1	Isoform 1 of Collagen alpha-1(I) chain precursor	69.99	0.74
19	Kpnb1	IPI00742334.1	Karyopherin (importin) beta 1	17.92	0.74
20	Hnrnpa1	IPI00553777.2	Heterogeneous nuclear ribonucleoprotein A1	68.90	0.75
21	Calr	IPI00123639.1	Calreticulin precursor	54.57	0.76
22	LOC100046745	IPI00625588.1	Similar to Tu translation elongation factor, mitochondrial	26.33	0.77
23	Pdia6	IPI00222496.3	Thioredoxin domain containing 7	21.80	0.77
24	Slc3a2	IPI00114641.2	CD98 heavy chain	37.97	0.77
25	Tln1	IPI00465786.3	Talin-1	33.57	0.77
26	Phb	IPI00133440.1	Prohibitin	44.49	0.77
27	Hdlbp	IPI00123379.1	Vigilin	29.02	0.79
28	Pabpc1	IPI00331552.4	Poly A binding protein, cytoplasmic 1	49.53	0.79
29	Acaa2	IPI00226430.2	3-ketoacyl-CoA thiolase, mitochondrial	42.32	0.79
30	Mdh2	IPI00323592.2	Malate dehydrogenase, mitochondrial	29.59	0.80
31	LOC672195	IPI00222419.5	Cytochrome c, somatic	79.05	0.80
32	Gnb2l1	IPI00317740.5	Guanine nucleotide-binding protein subunit beta-2-like 1	54.89	0.81
33	Atp5h	IPI00881799.1	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d	61.59	0.81
34	Gpi1	IPI00228633.7	Glucose-6-phosphate isomerase	26.16	0.81
35	Hspd1	IPI00308885.6	Isoform 1 of 60 kDa heat shock protein, mitochondrial	55.85	0.81
36	Vdac1	IPI00230540.1	Isoform Mt-VDAC1 of Voltage-dependent anion-selective channel protein 1	63.25	0.81
37	Rplp2	IPI00139795.2	60S acidic ribosomal protein P2	79.13	0.81
38	LOC674678	IPI00623776.3	Similar to histone H4	44.94	0.81
39	LOC100041245	IPI00851010.1	Similar to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	61.20	0.82
40	Bat1a	IPI00409462.2	Spliceosome RNA helicase Bat1	28.50	0.82
41	Dpysl2	IPI00114375.2	Dihydropyrimidinase-related protein 2	34.79	0.82
42	Eno1	IPI00462072.3	Enolase 1, alpha non-neuron	71.20	0.82
43	Immt	IPI00381412.1	Isoform 2 of Mitochondrial inner membrane protein	33.24	0.82
44	Sod2	IPI00109109.1	Superoxide dismutase [Mn], mitochondrial	47.30	0.83
45	Hnrnpa3	IPI00269662.1	Isoform 2 of Heterogeneous nuclear ribonucleoprotein A3	72.27	0.83
46	Got2	IPI00117312.1	Aspartate aminotransferase, mitochondrial	42.09	0.83
47	Vat1	IPI00126072.2	Synaptic vesicle membrane protein VAT-1 homolog	36.21	1.20
48	Actg2	IPI00266875.5	Smooth muscle gamma-actin	71.25	1.21
49	Ppib	IPI00135686.2	Peptidylprolyl isomerase B	57.87	1.21
50	EG667035	IPI00755353.2	Similar to fusion protein: ubiquitin (bases 43_513); ribosomal protein S27a	54.49	1.21
51	Iqgap1	IPI00467447.3	Ras GTPase-activating-like protein IQGAP1	28.85	1.22
52	Lasp1	IPI00125091.1	LIM and SH3 domain protein 1	25.48	1.22

53	Pfn1	IPI00224740.6	Profilin-1	48.57	1.22
54	LOC100045699	IPI00848492.1	Similar to Electron transferring flavoprotein, beta polypeptide isoform 2	36.82	1.24
55	Col6a3	IPI00877197.1	Collagen alpha3(VI) precursor (Fragment)	39.46	1.24
56	Flnb	IPI00663627.1	Filamin, beta	36.16	1.25
57	Gpnmb	IPI00311808.2	Transmembrane glycoprotein NMB precursor	41.64	1.25
58	Reversed	IPI00651986.1	7 days neonate cerebellum cDNA, hypothetical protein	14.75	1.26
59	Hsp90b1	IPI00129526.1	Endoplasmin precursor	38.78	1.26
60	Atp5c1	IPI00776084.1	ATP synthase gamma chain	48.15	1.26
61	Efhd2	IPI00226872.1	SWIPROSIN 1	35.00	1.28
62	Psap	IPI00321190.1	Sulfated glycoprotein 1 precursor	30.70	1.28
63	Phb2	IPI00321718.4	Phb2 Prohibitin-2	52.84	1.28
64	Tpi1	IPI00467833.5	Triosephosphate isomerase	54.62	1.28
65	Hsp90ab1	IPI00554929.2	Heat shock protein HSP 90-beta	45.03	1.29
66	Eif4a1	IPI00118676.3	Eukaryotic initiation factor 4A-I	42.61	1.31
67	Ncl	IPI00317794.5	Nucleolin	33.38	1.31
68	Anxa4	IPI00877291.1	Annexin A4	50.47	1.31
69	Anxa2	IPI00468203.3	Annexin A2	65.78	1.32
70	Tuba3b	IPI00466390.1	Tubulin alpha-3 chain	42.22	1.32
71	Sept7	IPI00874440.1	Septin-7	44.50	1.33
72	Cald1	IPI00462119.3	Caldesmon 1	57.30	1.34
73	Ran	IPI00134621.3	GTP-binding nuclear protein Ran	31.02	1.36
74	Hspa1a	IPI00798482.4	Heat shock 70 kDa protein 1A	35.26	1.37
75	Tpm4	IPI00421223.3	Tropomyosin alpha-4 chain	75.40	1.38
76	Cltc	IPI00648173.1	Clathrin, heavy polypeptide	22.28	1.38
77	Gm2a	IPI00119095.3	Ganglioside GM2 activator precursor	23.83	1.39
78	Tmpo	IPI00828976.1	Thymopoietin isoform epsilon	34.29	1.39
79	LOC632297	IPI00417181.3	UDP-glucuronosyltransferase 1-7C precursor	21.47	1.41
80	Blvrb	IPI00113996.7	Flavin reductase	57.77	1.46
81	Ckap4	IPI00223047.2	Cytoskeleton-associated protein 4	34.26	1.47
82	Actn1	IPI00380436.1	Alpha-actinin-1	38.45	1.47
83	P4ha1	IPI00399959.1	Isoform 2 of Prolyl 4-hydroxylase subunit alpha-1 precursor	35.96	1.49
84	Anxa6	IPI00310240.4	Annexin A6 isoform b	35.68	1.49
85	Sfrs6	IPI00310880.4	Splicing factor, arginine/serine-rich 6	54.57	1.50
86	Aldh2	IPI00111218.1	Aldehyde dehydrogenase, mitochondrial	35.84	1.51
87	Col6a2	IPI00621027.2	Collagen alpha-2(VI) chain precursor	36.85	1.53
88	Gsn	IPI00117167.2	Isoform 1 of Gelsolin precursor	43.85	1.53
89	Cat	IPI00869393.1	Catalase	39.47	1.56
90	Mrc1	IPI00126186.1	Macrophage mannose receptor 1 precursor	17.72	1.57
91	Dbn1	IPI00331516.3	Isoform E2 of Drebrin	21.33	1.61
92	Ahnak	IPI00553798.2	AHNAK nucleoprotein isoform 1	64.02	1.64
93	Arpc1b	IPI00874737.2	Actin related protein 2/3 complex, subunit 1B	36.83	1.66
94	Hk2	IPI00114342.1	Hexokinase-2	33.81	1.67
95	Tpm1	IPI00403987.2	Tropomyosin 1, alpha	76.76	1.71
96	LOC623483	IPI00660661.2	Similar to ribosomal protein L22	61.78	1.71
97	Hspg2	IPI00515360.8	Perlecan	28.41	1.72
98	Basp1	IPI00129519.3	Brain acid soluble protein 1	66.81	1.74
99	Oat	IPI00129178.1	Ornithine aminotransferase, mitochondrial	21.87	1.87
100	Krt8	IPI00322209.5	Keratin, type II cytoskeletal 8	58.57	1.89
101	Snx2	IPI00109212.3	Sorting nexin-2	20.04	1.98
102	Arhgdib	IPI00122568.3	Rho GDP-dissociation inhibitor 2	25.00	1.98
103	Lrp1	IPI00119063.2	Prolow-density lipoprotein receptor-related protein 1 precursor	27.24	2.13
104	Hspa9	IPI00880839.1	Heat shock protein 9	53.61	2.63
105	Krt19	IPI00112947.1	Keratin, type I cytoskeletal 19	67.74	2.94
106	Fn1	IPI00652813.1	Fibronectin 1	53.20	3.73
107	Gusb	IPI00421209.3	Glucuronidase, beta	37.96	4.74

Table 2. Seventy-nine proteins common in 2 of 3 groups (7co/3co, 7co/7d, 3co/3d) were listed

Upregulated gene symbol and protein name	
Actg2	Smooth muscle gamma-actin
Ppib	Peptidylprolyl isomerase B
Iqgap1	Ras GTPase-activating-like protein IQGAP1
Pfn1	Profilin-1
Col6a3	Collagen alpha3(VI) precursor (Fragment)
Tpi1	Triosephosphate isomerase
Anxa4	Annexin A4
Tuba3b	Tubulin alpha-3 chain
Sept7	Septin-7
Hspa1a	Heat shock 70 kDa protein 1A
Gm2a	Ganglioside GM2 activator precursor
Tmpo	Thymopoietin isoform epsilon
LOC632297	UDP-glucuronosyltransferase 1-7C precursor
Blvrb	Flavin reductase
Actn1	Alpha-actinin-1
P4ha1	Isoform 2 of Prolyl 4-hydroxylase subunit alpha-1 precursor
Sfrs6	Splicing factor, arginine/serine-rich 6
Aldh2	Aldehyde dehydrogenase, mitochondrial
Col6a2	Collagen alpha-2(VI) chain precursor
Mrc1	Macrophage mannose receptor 1 precursor
Dbn1	Isoform E2 of Drebrin
Ahnak	AHNAK nucleoprotein isoform 1
Arpc1b	Actin related protein 2/3 complex, subunit 1B
LOC623483	Similar to ribosomal protein L22
Basp1	Brain acid soluble protein 1
Arhgdib	Rho GDP-dissociation inhibitor 2
Hsp90b1	Endoplasmin precursor
Psap	Sulfated glycoprotein 1 precursor
Cltc	Clathrin, heavy polypeptide
Gusb	Glucuronidase, beta
Atp5b	ATP synthase subunit beta, mitochondrial
Cisd1	CDGSH iron sulfur domain-containing protein 1
Eif5a	Eukaryotic translation initiation factor 5A-1
Myl6	Isoform Smooth muscle of Myosin light polypeptide 6
Ywhaz	14-3-3 protein zeta/delta
Eif2s1	Eukaryotic translation initiation factor 2 subunit 1
Pgk1	Phosphoglycerate kinase 1
LOC100041245	Similar to Glyceraldehyde-3-phosphate dehydrogenase(GAPDH)
Scpep1	Serine carboxypeptidase 1
Sod1	Superoxide dismutase
Bat1a	Spliceosome RNA helicase Bat1
Tagln2	Transgelin 2
Downregulated gene symbol and protein name	
Nes	Isoform 1 of Nestin
Snrpb	Small nuclear ribonucleoprotein-associated protein B
Sfrs7	Isoform 1 of Splicing factor, arginine/serine-rich 7
Hist3h2a	Histone H2A type 3
Hnrnpab	Heterogeneous nuclear ribonucleoprotein A/B

mM KH_2PO_4 in 25% ACN, pH2.6) at a flow rate of 200 μL/min for 90 min, and 30 fractions were collected every 3 min. Each fraction was dried down and redissolved in buffer C (5% (v/v) acetonitrile and 0.1% formic acid solution), and the fractions with high KCl concentration were desalted with PepClean C-18 spin Column (Pierce, USA).

DLC-ESI-MS/ MS

All SCX fractions were analysed 3 times using a QSTAR XL LC-MS/MS system (Applied Biosystems, USA) and RPLC column (ZORBAX 300SB-C18 column, 5 μm, 300 Å, 0.1 mm × 15 mm, Microm, Auburn, CA). The RPLC gradient was 5% to 35% buffer D (95% acetonitrile, 0.1% formic acid) in buffer C at a flow rate of 0.3 μL/min in 120 min.

The Q-TOF instrument was operated in positive ion mode with ion spray voltage typically maintained at 2.0 kV. Mass spectra of the iTRAQ-labelled samples were acquired in an information-dependent acquisition mode. The analytical cycle consisted of a MS survey scan (400-2000 m/z) followed by 5-s MS/MS scans (50-2000) of the 5 most abundant peaks (i.e., precursor ions), which were selected from the MS survey scan. Precursor ion selection was based upon ion intensity (peptide signal intensity

Sfpq	Splicing factor, proline- and glutamine-rich
Ppia	Peptidyl-prolyl cis-trans isomerase
P4hb	Protein disulfide-isomerase precursor
EG666548	Similar to ribosomal protein L23a
Kpnb1	Karyopherin (importin) beta 1
Slc3a2	CD98 heavy chain
Tln1	Talin-1
Acaa2	3-ketoacyl-CoA thiolase, mitochondrial
LOC672195	Cytochrome c, somatic
Eno1	Enolase 1, alpha non-neuron
Immt	Isoform 2 of Mitochondrial inner membrane protein
Sod2	Superoxide dismutase [Mn], mitochondrial
Got2	Aspartate aminotransferase, mitochondrial
Dync1h1	Cytoplasmic dynein 1 heavy chain 1
Rplp2	60S acidic ribosomal protein P2
LOC674678	Similar to histone H4
Hnrnpa3	Isoform 2 of Heterogeneous nuclear ribonucleoprotein A3
Krt19	Keratin, type I cytoskeletal 19
Krt8	Keratin, type II cytoskeletal 8
Hsp90ab1	Heat shock protein HSP 90-beta
Samm50	Sorting and assembly machinery component 50 homolog
Abhd12	Abhydrolase domain-containing protein 12
D1Pas1	Putative ATP-dependent RNA helicase PI10
Dci	Dodecenoyl-Coenzyme A delta isomerase
Glud1	Glutamate dehydrogenase 1, mitochondrial
Vdac2	Voltage-dependent anion-selective channel protein 2
Gsn	Isoform 1 of Gelsolin precursor
Hist2h2ac	Histone H2A type 2-C
LOC675857	Similar to valosin isoform 1
Prdx1	Peroxiredoxin-1
Calm3	Calmodulin 3
Flnb	Filamin, beta

above 25 counts/s) and charge state (2+ to 4+), and once the ions were fragmented in the MS/MS scan they were allowed 1 repetition before a dynamic exclusion for a period of 120 s. Because of the iTRAQ tags, the parameters for rolling collision energy (automatically set according to the precursor m/z and charge state) were manually optimised. Under CID, iTRAQ-labelled peptides fragmented to produce reporter ions at 115.1, 116.1, 117.1, and 118.1, and fragment ions of the peptides were simultaneously produced, resulting in sequencing of the labelled peptides and identification of the corresponding proteins. The ratios of the peak areas of the four iTRAQ reporter ions reflected the relative abundances of the peptides and the proteins in the samples. Calibration of the mass spectrometer was carried out using BSA tryptic peptides.

Protein identification and data analysis

Protein identification and quantification for iTRAQ experiments was carried out using the ProteinPilot software v3.0 (Applied Biosystems, USA). The search was performed against an International Protein Index (IPI) mouse database (version 3.28) downloaded from the EBI Web site. The Paragon algorithm in ProteinPilot software was used as the default search program with trypsin as the digestion agent and cysteine modification of methyl methanethiosulfonate. The search also included the possibility of more than 80 biological modifications and amino acid substitutions of up to two substitutions per peptide using the BLOSUM 62 matrix. Only proteins identified with at least 95% confidence, or a ProtScore of 1.3, were reported. The results obtained from ProteinPilot v3.0 software were exported to Microsoft Excel and Microsoft Access for further analysis.

A 1.2-fold change (ratio of BMSCs co-cultured for 7 days to BMSCs co-cultured for 3 days, i.e.,7co/3co) was used as the benchmark. All proteins that showed significantly altered expression levels went through Ingenuity Pathway Analysis software (IPA) for pathway and network analysis.

Results

BMSCs and EStCs primary culture and characterisation

CD29 is one of mesenchymal stem cell surface markers and CD34 is one of haemopoietic stem cell markers [13, 20, 21]. The flow cytometry analysis showed that the 81.22% of BMSCs

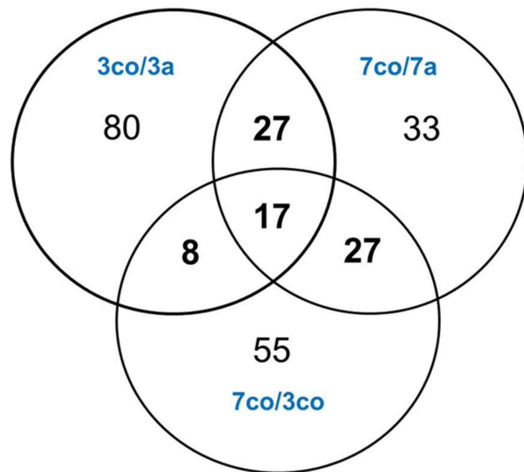

Figure 3. Differentially expressed proteins in 7co/3co (118:116), 7co/7a (118:117) and 3co/3a (116:115) groups were diagrammed and common proteins were marked in bold.

were positive for CD29, and 20.86% of BMSCs were positive for CD34 (**Figure 1B**). Cultured EStCs were confirmed by the immunocytochemistry experiments to show that they were positive for the stromal cell marker vimentin (**Figure 1C**). By culture alone and co-culture, samples from the following four groups were acquired: Group 1: BMSCs cultured alone for 3 days (3a), Group 2: BMSCs co-cultured for 3 days (3co), Group 3: BMSCs cultured alone for 7 days (7a) and Group 4: BMSCs co-cultured for 7 days (7co).

Differential proteins identified in differentiation by iTRAQ proteomics

Samples from all 4 groups were digested, quantified and underwent iTRAQ proteomics. A total of 9932 peptides were identified, 6549 of which were unique. These identified peptides correspond to a set of 311 proteins with more than 95% confidence (ProtScore ≥ 1.3). Of 213 proteins that were identified with a global false discovery rate from fit values of 1%, 210 proteins were identified with relative quantitation (Supplementary Table 1).

According to the ratio of BMSCs co-cultured for 7 days (7co) to those co-cultured for 3 days (3co), 107 proteins were differentially expressed, with 61 up-regulated and 46 down-regulated proteins (**Table 1**). According to the ratio of BMSCs co-cultured for 7 days (7co) to those cultured alone for 7 days (7a), 104 proteins were differentially expressed, with 56 up-regulated and 48 down-regulated proteins (Supplementary Table 2). According to the ratio of BMSCs co-cultured for 3 days (3co) to those cultured alone for 3 days (3a), 132 proteins were differentially expressed, with 75 up-regulated and 57 down-regulated proteins (Supplementary Table 3). In the 3 groups above (7co/3co, 7co/7d, 3co/3d), 17 proteins are common in 3 groups and 79 proteins are common in 2 groups (**Table 2**, **Figure 3**).

Bioinformatic analysis of differential proteins

To interpret the alterations in the differentiation process, we used IPA to analyse and acquired canonical pathways and top networks based on differentially expressed proteins. In the 107 differential proteins in 7co/3co group, the top 10 canonical pathways were "remodeling of epithelial adhesions junctions", "actin cytoskeleton signaling", "epithelial adhesions junction signaling", "germ cell-Sertoli cell junction signaling", "glycolysis I", "glyconeogenesis I", "superoxide radicals degradation", "aspartate degradation II", "regulation of actin-based motility by Rho", "aldosterone signaling in epithelial cells", "phenylalanine degradation IV" (**Figure 4**). In the 79 common proteins in 2 groups, the top 10 canonical pathways were "actin cytoskeleton signaling", "remodeling of epithelial adherens junctions", "regulation of actin-based motility by Rho", "epithelial adhesions junction signaling", "glyconeogenesis I", "RhoA signaling", "superoxide radicals degradation", "germ cell-sertoli cell junction signaling", "NRF2-mediated oxidative stress response", "phenylalanine degradation IV" (**Figure 5**).

The top 4 networks that differential proteins in 7co/3co group participated in were the "cell-to-cell signaling and interaction, tissue development and cellular movement" network (score = 28), the "cell morphology, nerve development and function, organ morphology" network (score = 24), the "cell morphology, cellular function and maintenance, cell death and survival" network (score = 20), and the "amino acid metabolism, small molecule biochemistry, protein synthesis" network (score = 20) (**Table 3**). Among them, core proteins of the network 1 (**Figure 6**) were Fn1, Col1A1, Lrp1, Hsp90b1, which were regulated by ERK1/2, ERK and α-catenin.

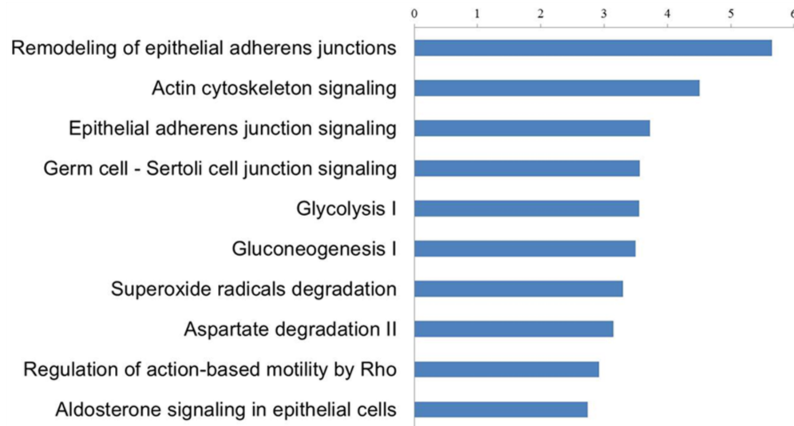

Figure 4. Top 10 canonical pathways that differential proteins in 7co/3co group participated in were illustrated.

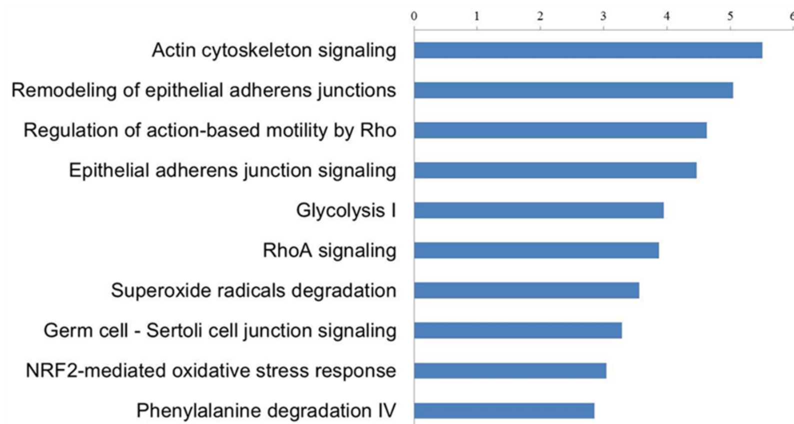

Figure 5. Top 10 canonical pathways that 79 common proteins participated in were illustrated.

Discussion

Primary culture of BMSCs and EStCs provided the basis for iTRAQ proteomics in 4 groups. In order to reveal potential changes in the differentiation process, we compared proteins quantity in 7co group with 3co group (7co/3co).

Nes was the most significantly regulated differential protein with a 20-fold change (ratio 0.05) in expression. Nes is a type VI intermediate filament protein that is expressed in dividing cells during the early stages of development in the central nervous system, peripheral nervous system and in myogenic and other tissues [22, 23]. In depth studies of Nes indicate that it plays a complex role in the regulation of the assembly and disassembly of intermediate filaments that participate in cell remodelling [23]. Nes is a marker of proliferating and migrating cells and becomes down-regulated upon differentiation [23, 24]. In our study, Nes was most significantly down-regulated protein, showing that co-cultured BMSCs significantly differentiated and their proliferating and migrating ability substantially decreased, which was in accordance withtheir differentiation into EECs. Epithelial marker CK19, CK8 were both upregulated and listed in differential proteins (ratio 2.94, 1.89 respectively, **Table 1**). Stromal marker vimentin were down-regulated (ratio 0.90). Both the increase of CK19, CK8 and the decrease of vimentin demonstrated that BMSCs differentiated in the direction of EECs in the co-culture system.

The top 20 canonical pathways showed amazing consistence in 7co/3co and common differential proteins groups. In both groups, the "remodeling of epithelial adhesions junctions" and "actin cytoskeleton signaling" were the top 2 canonical pathway. In addition, "epithelial adhesions junction signaling", "germ cell-sertoli cell junction signaling", "regulation of actin-based motility by Rho" are another 3 canonical pathways. All these canonical pathways implicated that epithelial adhesion junction and motility were significantly regulated.

In the top network, ERK1/2 played important roles by regulating core proteins, including Fn1, Col1a1. ERK1 was also known as MAPK3; ERK or ERK2 was known as MAPK1. The ERK (extracellular-regulated kinase)/MAPK (mitogen activated protein kinase) pathway is a key pathway that transduces cellular information on meiosis/mitosis, growth, differentiation and carcinogenesis within a cell. ERK in the cytoplasm can phosphorylate a variety of targets which include

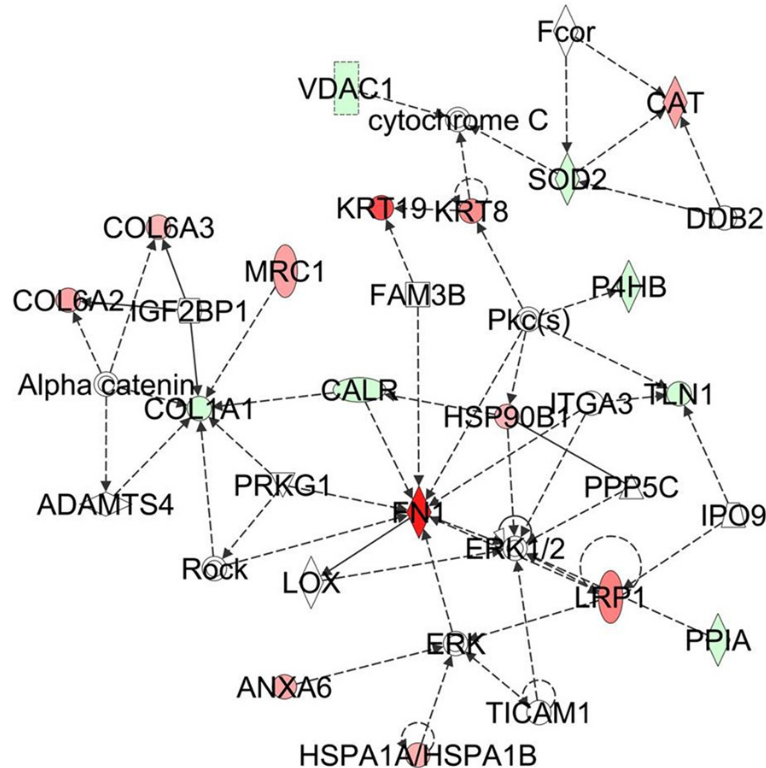

Figure 6. Top network that differential proteins in 7co/3co group participated in was "cell-to-cell signaling and interaction, tissue development, cellular movement". Core proteins, i.e., Fn1, Col1A1, Lrp1, Hsp90b1 were regulated by ERK1/2, ERK and α-catenin.

cytoskeleton proteins, ion channels/receptors and translation regulators. Fn1 (fibronectin 1) is a glycoprotein present in a soluble dimeric form in plasma, and in a dimeric or multimeric form at the cell surface and in extracellular matrix. It is involved in cell adhesion and migration processes and canonical pathway "actin cytoskeleton signaling" [25, 26]. In our study, Fn1 was the 2nd most significantly upregulated differential protein (ratio 3.73) regulated by ERK1/2, which demonstrated increased cell adhesion and decreased migration in "actin cytoskeleton signaling" canonical pathway. Col1a1 (Isoform 1 of Collagen alpha-1(I) chain precursor), is the fibrillar collagen found in most connective tissues. In our study, Col1a1 were significantly downregulated (ratio 0.74) demonstrated the stromal component decreased in condition of co-culture, which indicated that BMSCs tended to differentiate into EECs in the co-culture system.

To the best of our knowledge, this is the first study of protein alterations in the differentiation process of BMSCs in the direction of EECs in vitro. Both epithelial marker and stromal marker expression alteration further implicated the differentiation process. Our study also indicated that ERK/MAPK might play potential important roles by regulating core differential proteins in the "cell-to-cell signaling and interaction, tissue development and cellular movement" network.

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Disclosure of conflict of interest

None.

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