SFRP1 and PTGS2 are potential biomarkers of tumorigenic properties in human placenta-derived mesenchymal stem cells

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Abstract

Background

Mesenchymal stem cells (MSCs) display tumour tropism and have been explored as cellular vehicles to deliver anti-cancer agents. As cellular components of the tumour microenvironment, MSCs also influence tumour progression. However, the tumour transformation-related genes of MSCs are not well understood since either oncogenic or tumour suppressor effects within these cells have been reported. Here, we aimed to identify potential biomarkers capable of tumorigenic risk by RNA-seq analysis of human placenta tissue-derived MSCs (hPTMSCs) exposed to the carcinogenic agent 3-methylcholanthrene (3-MC).

Results

Twenty-nine tumour transformation-related genes and three pluripotency-related genes were identified as differentially expressed genes (DEGs) in hPTMSCs. Importantly, SFRP1 and PTGS2 were further identified as tumour suppressors and oncogenes in hPTMSCs, respectively. The overexpression of SFRP1 and PTGS2 was positively correlated with the expression of the pluripotent markers OCT4, CMYC and NANOG in the human lung adenocarcinoma cell line A549, and the low expression of SFRP1 and PTGS2 was inversely correlated with the expression of the pluripotent markers SOX2, OCT4 and NANOG in hPTMSCs. Interestingly, overexpression of SFRP1 led to reduced cell viability, colony formation and migration of A549 cells. In contrast, the ectopic expression of PTGS2 exerted the opposite effect.

Conclusions

These results indicate that hPTMSCs with high PTGS2 expression but low SFRP1 expression may have more potential tumorigenic capacity. Taken together, this study suggests that PTGS2 and SFRP1 may be valuable biomarkers for quality and safety control of hPTMSC preparations in clinical applications, which warrants further study.

Background

Mesenchymal stem cells (MSCs) are excellent candidates for a diverse range of clinical applications in tissue regeneration, cell therapy and immunomodulation[1]. Their characteristics include plastic adherence; trilineage differentiation capacity into osteoblasts, chondrocytes and adipocytes in vitro; and a surface phenotype defined by the absence of haematopoietic and antigen-presenting surface markers and the presence of MSC-characteristic antigens CD73, CD90, and CD105[2]. MSCs can be found in various tissues, including bone marrow, cord blood, adipose tissue and placentas[3]. Among these sites, human placental tissue is accessible and abundant for the isolation of adult stem cells. Therefore, human placenta tissue-derived MSCs (hPTMSCs) have become an attractive source for cell therapy. Ra JC et al reported the safety of intravenous infusion of human adipose tissue-derived MSCs (hATMSCs) in animals and humans[4]. MacIsaac et al also showed that no tumorigenic properties were detected in mice treated with adipose stromal/stem cells[5]. However, in tumour models, MSCs have been reported to play a role in the process of carcinogenesis, tumour growth and metastasis, although numerous studies have reported contradictory results; some investigators found that MSCs promote

tumour growth, and others reported that MSCs inhibit tumour growth[6–8]. Zhang T et al reported that bone marrow-derived MSCs (BM-MSCs) promote the growth and angiogenesis of breast and prostate tumours[9]. Rodini et al. also found that MSCs enhanced the tumorigenic properties of human glioblastoma through independent cell-cell communication mechanisms[10]. Nevertheless, Qiao L et al. reported that Dkk-1 secreted by MSCs inhibits the growth of breast cancer cells via the depression of Wnt signalling[11]. Yin J also found that MSC-mediated concurrent delivery of endostatin and carboxylesterase to mouse xenografts suppresses glioma initiation and recurrence[12]. Some mechanisms of MSC-related tumour supporting effects have been reported, including vascular[9, 13, 14] and tumour microenvironment support, immunosuppressive effects and metastatic support[15–17]. On the other hand, the tumour inhibitory mechanisms of MSCs could be due to cell cycle arrest[18, 19], angiogenesis inhibition[20] and regulation of certain soluble factors, such as the Wnt inhibitor DKK-1, that may block tumour-related cell signalling pathways[21]. Despite these explanations regarding the action of MSCs, the tumour modulation mechanisms of MSCs are extremely complicated and difficult to demonstrate. Thus, the potential tumorigenicity of MSCs should be further explored and monitored to elucidate the risk of potential tumorigenicity related to MSC-based therapies[22].

The accumulation of mutations, genetic predisposition of some factors, exposure to environmental agents, and lifestyle all play important roles in the carcinogenic process. 3-Methylcholanthrene (3-MC), a potent polycyclic aromatic hydrocarbon (PAH), is a ubiquitous environmental pollutant that can be obtained as a by-product of combustion of various organic substances, such as coal, garbage, gas and oil[23]. MC and other PAHs have many untoward effects, including immune suppression, endocrine disruption, wasting syndrome, birth defects and carcinogenesis[24, 25]. These compounds suppress the proliferation of human umbilical vascular endothelial cells and induces tumours in mice and rats[26].

Here, we aimed to identify potential biomarkers capable of tumorigenic risk by RNA-seq analysis of hPTMSCs exposed to the carcinogenic agent 3-MC and provide markers for detecting tumorigenicity related to quality and safety control of clinical-grade MSCs in cell therapy.

Results

Sequencing data and quality control

RNA samples were extracted from DMSO control and 3-MC-treated hPTMSCs using TRIzol. The Illumina HiSeq 2500 RNA sequencing platform was used to sequence the whole transcriptome of hPTMSCs. Raw data (raw reads) in FASTQ format were first processed. In this step, clean data (clean reads) were obtained by removing reads containing adapters and low-quality reads from raw data. In addition, the Q20, Q30, GC content and sequence duplication level of the clean data were calculated. All downstream analyses were based on clean data with high quality. After stringent data filtering and quality checks, approximately 62 million high-quality clean reads were obtained from both samples with 87.11% and 86.44% Q30 bases (base quality was more than 30) for DMSO control and 3-MC treated hPTMSCs, respectively. In total, 29,251,305 and 32,739,136 clean paired-end reads as well as 8,730,066,236 and 9,771,496,192 clean bases were generated from DMSO control and 3-MC treated hPTMSCs, respectively (Table 1). These clean reads were then mapped to the reference genome sequence. Only reads with a perfect match or mismatch were further analysed and annotated based on the reference genome. Tophat2 tools software was used to map these reads based on the reference genome.

Table 1
Statistical table of sequencing data

Samples	Clean reads	Clean bases	GC content	%≥Q30
DMSO control	29,251,305	8,730,066,236	55.81%	87.11%
3-Methylcholanthrene(MC)	32,739,136	9,771,496,192	55.80%	86.44%

Differentially expressed unigene analysis

Unigene expression was calculated by FPKM (fragments per kilobase of transcript per million fragments mapped)[27] using Cuffquant and Cuffnorm. Differential expression analysis was determined by EBSeq. The threshold of the false discovery rate (FDR) was set to less than 0.01 and with a fold change greater than or equal to 2. Through this calculation, the upregulation and downregulation of both DMSO control- and 3-MC-treated transcripts were determined. Ultimately, 1081 differentially expressed genes (DEGs) were identified, including 498 upregulated and 583 downregulated genes (Fig. 1A and 1B). Hierarchical cluster analysis of DEGs is presented in Fig. 1C. To further study how hPTMSCs regulate their biological functions in response to 3-MC at the molecular level, we focused on tumour transformation-related and pluripotency-related DEGs. A total of 268 tumour transformation-related genes were identified: 17 upregulated (C1QTNF1, CD14, CHI3L1, CXCL8, CXCL16, GATA3, GCH1, IRAK3, MN1, PID1, PLXDC2, PTGS2, PTK2B, TNFAIP3, TNFAIP6, TNFAIP8 and TNFSF13B) and 12 downregulated (IL18, KRT18, MTUS1, MTUS2, PRAME, PTTG1, SFRP1, TACSTD2, TLR4, TNFAIP8L1, TNFRSF19 and TXNIP) (Fig. 2A, 2C). In addition, 66 pluripotency-related genes were identified, including JAG1, LFNG and MAML3, which were downregulated in the Notch signalling pathway (Fig. 2B, 2C).

Functional annotation, gene ontology classification and pathway analysis

Functional annotation provided information on protein function annotation, pathway annotation, COG annotation (Clusters of Orthologous Groups of proteins) and GO annotation (Gene Ontology). Unigenes were subjected to sequence alignment with protein databases, such as NR (non-redundant protein sequence database), Swiss-Prot (a manually annotated, non-redundant protein sequence database), GO, KOG (Clusters of Protein homology), Pfam (Homologous protein family) and KEGG (Kyoto Encyclopedia of Genes and Genomes) using BLAST software, which is based on sequence similarities to public databases. In total, 1077 DEGs were successfully annotated. All DEGs were annotated using the NR (1077) followed by eggNOG (1063), Swiss-Prot (1055), Pfam (996), GO (948), KEGG (709), KOG (695) and COG databases (325). The remaining DEGs had no matches. The GO database is an international standard biological annotation system with three ontologies: biological process, cellular component and molecular function[28]. The GO classification of DEGs is presented in Fig. 3A. Pathway-based analysis provides information and further understanding on how hPTMSCs regulate their biological functions in response to 3-MC at the molecular level. KEGG is a database resource for understanding the high-level functions and utilities of biological systems, such as cells, organisms and ecosystems, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies. We used KOBAS software to test the statistical enrichment of DEGs in KEGG pathways. DEGs in the same pathways typically cooperate with each other to exercise their biological functions. In total, 865 DEGs were mapped to the KEGG database. These DEGs were classified into 50 KEGG pathways of four main KEGG categories, including cellular processes (129 DEGs), environmental information

processing (294 DEGs), human diseases (343 DEGs) and organismal systems (99 DEGs) (Fig. 3B). Out of 50 pathways, the first 20 pathways (Q value ≤ 1.00) are presented in Fig. 3C.

Verification of tumour transformation-related and pluripotency-related DEGs

To verify the results of tumour transformation-related and pluripotency-related DEGs in RNA sequencing, we first characterized the expression of these DEGs by qPCR. The expression trend of 27 tumour transformation-related DEGs as well as 3 pluripotency-related DEGs in the Notch signalling pathway exhibited consistency with the RNA sequencing, whereas the other 2 tumour transformation-related DEGs did not change (Fig. 4A). Among these genes, it has been reported that the downregulation of SFRP1 can lead to the overactivation of Wnt signalling pathways, which promote tumorigenesis in human mammary tissues[29, 30]. In addition, NF- κ B-mediated inflammation-driven carcinogenesis involves transactivation of prostaglandin-endoperoxide synthase 2 (PTGS2) [31]. PTGS2 is upregulated in various premalignant and malignant tissues[31]. Abnormally high PTGS2 levels contribute to tumorigenesis in various animal models of cancer[32–34]. More recently, genomic studies performed on MSCs have provided evidence that these cells express the pluripotent markers Oct-4, Nanog, and Sox2, which clarified their undifferentiated state[35]. To elucidate the possible roles of SFRP1 and PTGS2 in the 3-MC-induced tumorigenesis of hPTMSCs, gain- and loss-of-function studies were performed in hPTMSCs and the human lung adenocarcinoma cell line A549. Stable SFRP1 and PTGS2 overexpression or silencing in hPTMSCs and A549 cells were achieved using the respective lentiviruses. QPCR and western blotting verified that SFRP1 overexpression and silencing in hPTMSCs was accompanied by a significant decrease in CMYC and an increase in SOX2 and NANOG. PTGS2 overexpression and silencing was accompanied by a significant decrease in CMYC and an increase in SOX2 (Fig. 4B and 4C). Moreover, SFRP1 overexpression and silencing in A549 cells were accompanied by a significant decrease in SOX2 and an increase in OCT4 and NANOG, and PTGS2 overexpression and silencing were accompanied by a significant decrease in SOX2 and an increase in OCT4 and NANOG (Fig. 4D and 4E).

Additionally, CCK-8, colony-formation and wound healing assays showed that overexpression of SFRP1 led to reduced cell viability, colony formation and migration of A549 cells. In contrast, the ectopic expression of PTGS2 resulted in significantly increased cell viability, colony formation and migration of A549 cells. These results indicate that hPTMSCs with high PTGS2 expression but low SFRP1 expression may have more potential tumorigenic capacity; cells with low PTGS2 expression and high SFRP1 expression may exhibit less tumorigenic capacity. Taken together, these results suggest that PTGS2 and SFRP1 may represent valuable biomarkers for the quality and safety control of hPTMSC preparations in clinical applications, which warrants further study (Fig. 5).

Discussion

MSCs are multipotent, self-renewing cells with the capacity to differentiate into cells of mesenchymal origin, including osteoblasts, chondrocytes, and adipocytes in vitro and give rise to bone, cartilage, fat and muscle tissues in vivo. Their differentiation potential together with their ease of isolation and cultivation has made them a potentially important source of clinical application in tissue engineering and regenerative medicine.

Clinically, MSCs may aid in the reconstitution of tissues as well as the maintenance of tissue homeostasis, which brings about some concern regarding the potential tumorigenicity of MSC-based therapies[36]. Although some

studies suggest that MSCs have inhibitory effects on tumour development, an overwhelming number of studies demonstrate that MSCs exert stimulatory effects on tumour pathogenesis[22]. Djouad et al. showed that MSCs favour earlier onset and growth of syngenic tumours in allogenic animals given their immunosuppressive effects[37, 38].

Polycyclic aromatic hydrocarbons (PAHs), including 3-MC, are ubiquitous environmental pollutants that have many untoward effects, including carcinogenesis. In the present study, we aimed to identify potential biomarkers of tumorigenic risk by RNA-seq analysis of hPTMSCs exposed to 3-MC. In total, 268 tumour transformation-related genes of MC-induced MSCs were identified by RNA-seq, including 17 upregulated (C1QTNF1, CD14, CHI3L1, CXCL8, CXCL16, GATA3, GCH1, IRAK3, MN1, PID1, PLXDC2, PTGS2, PTK2B, TNFAIP3, TNFAIP6, TNFAIP8 and TNFSF13B) and 12 downregulated (IL18, KRT18, MTUS1, MTUS2, PRAME, PTTG1, SFRP1, TACSTD2, TLR4, TNFAIP8L1, TNFRSF19 and TXNIP) genes. In addition, 66 pluripotency-related genes were identified, including 3 downregulated Notch signalling pathway genes: including JAG1, LFNG and MAML3. We then characterized the expression of these DEGs by qPCR and found that the expression trends of 27 tumour transformation-related DEGs as well as 3 pluripotency-related DEGs in the Notch signalling pathway were consistent with the RNA sequencing results; the other 2 tumour transformation-related DEGs did not change.

Secreted Frizzled-related protein 1 (SFRP1), which is located within the 8p11-12 region, is homologous to the extracellular cysteine-rich domain of the wingless and integration site growth factor (WNT) receptor Frizzled but lacks the intracellular and transmembrane domains[39]. It has been reported that the downregulation of SFRP1 can lead to the overactivation of Wnt signalling pathways, which promotes tumorigenesis in human mammary tissues[29, 30]. A recent focus in cancer prevention involves the modulation of molecular cross-talk between inflammation and cancer growth signalling networks converging on the redox-sensitive transcription factor nuclear factor- κ B (NF- κ B). NF- κ B is directly associated with inflammatory responses with further links to the promotion of carcinogenesis[40–43]. NF- κ B-mediated inflammation-driven carcinogenesis involves transactivation of a major molecular target, prostaglandin-endoperoxide synthase 2 (PTGS2)[31]. PTGS2 is involved in prostaglandin (PG) biosynthesis and inflammation and is upregulated in various premalignant and malignant tissues[31]. Abnormally high levels of PTGS2 contribute to tumorigenesis in various animal models of cancer, and downregulation of PTGS2 is spontaneously preventive[32–34]. Various stimuli and mediators are involved in transient induction of NF- κ B and transactivation of PTGS2, thus eliciting inflammation and consequent tumorigenesis[31–34, 41]. Thus, targeted inhibition of NF- κ B and PTGS2 has become a promising and practical tool in cancer prevention. Reports suggest that diminishing NF- κ B-PTGS2 crosstalk may prevent tumorigenesis. More recently, genomic studies performed on MSCs demonstrate that these cells express the pluripotent markers Oct-4, Nanog, and Sox2, clarifying their undifferentiated state[35].

Given this finding, gain- and loss-of-function studies were performed in hPTMSCs and the human lung adenocarcinoma cell line A549. QPCR and western blotting verified that SFRP1 overexpression and silencing in hPTMSCs was accompanied by a significant decrease in CMYC and an increase in SOX2 and NANOG expression. PTGS2 overexpression and silencing in hPTMSCs was accompanied by a significant decrease in CMYC and an increase in SOX2 expression. Moreover, SFRP1 overexpression and silencing in A549 cells was accompanied by a significant decrease in SOX2 and an increase in OCT4 and NANOG expression, and PTGS2 overexpression and silencing in A549 cells was accompanied by a significant decrease in SOX2 and an increase in OCT4 and NANOG expression. Additionally, CCK-8, colony-formation and wound healing assays showed that overexpression of

SFRP1 led to reduced cell viability, colony formation and migration of A549 cells. In contrast, the ectopic expression of PTGS2 exerted the opposite effect.

Conclusions

In our study, 29 tumour transformation-related genes and 3 pluripotency-related genes were identified as differentially expressed genes (DEGs) in hPTMSCs exposed to 3-MC based on RNA-seq transcriptional profile analysis using functional annotation, Gene Ontology classification and pathway analysis. Importantly, SFRP1 and PTGS2 were further identified as tumour suppressors and oncogenes in hPTMSCs, respectively. Interestingly, overexpression of SFRP1 led to a reduced cell viability, colony formation and migration of A549 cells. In contrast, the ectopic expression of PTGS2 exerted the opposite effect. These results indicate that hPTMSCs with high PTGS2 expression but low SFRP1 expression may have more potential tumorigenic capacity. Cells with low PTGS2 expression and high SFRP1 expression may have less tumorigenic capacity. Taken together, this study suggests that PTGS2 and SFRP1 may be valuable biomarkers for quality and safety control of clinical-grade hPTMSC preparations, which warrants further study.

Methods

Cell Culture and treatment

The collection of human placentas was approved by the Ethics Committee of Human Research at Ningxia Medical University. Written consent was obtained from each mother prior to delivery. Three human full-term placentas were obtained from healthy mothers after elective caesarean section in the General Hospital of Ningxia Medical University. Human PTMSCs were isolated as described previously[44]. Cells were cultured in serum-free medium containing MesenCult-XF Basal Medium and MesenCult-XF Supplement (STEMCELL Technologies Inc., Grenoble, France). At about 90% confluence, cells were dissociated using the MesenCult-ACF Dissociation Kit (STEMCELL Technologies Inc., Grenoble, France) and passaged. The hPTMSCs at passage 3 were treated with 3-MC (SIGMA-ALDRICH, Saint Louis, USA) at a concentration of 5 µg/ml for serial 7days to induce carcinogenesis. All the studies were conducted with five passages of the isolated hPTMSCs. Human lung adenocarcinoma cell line A549 were purchased from the Cell Resource Center of the Chinese Academy of Sciences (Shanghai, China). A549 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA). Cells were harvested to conduct assays while confluence reached about 90% using TrypLE™ Express (Invitrogen, USA). All cultures were maintained at 37 °C in a sterile humidified incubator with 5% CO₂.

Flow cytometry

The immunological characterization of hPTMSCs was analyzed by flow cytometry on a FACS Calibur flow cytometer (BD Biosciences, San Diego, CA, USA). All monoclonal antibodies used for flow cytometry were purchased from BD Pharmingen (Franklin Lakes, NJ, USA). Briefly, cells (1×10^6) in 100 µl PBS were incubated with one of the following antibodies for 30 minutes at room temperature: IgG1-PE, CD34-PE, CD73-PE, IgG2a-FITC, CD14-FITC, CD45-FITC, CD90-FITC, CD105-FITC or HLA-DR-FITC. After extensive washing, cells were resuspended in 500 µL PBS and used for flow cytometry analysis.

RNA extraction and sequencing

Total RNA was extracted from DMSO control and 3-MC treated hPTMSCs using TRIzol Reagent (Ambion, America). RNA degradation and contamination were monitored on 1% agarose gels. RNA purity was checked using the NanoPhotometer spectrophotometer (IMPLEN, CA, USA). RNA concentration was measured using Qubit RNA Assay Kit in Qubit 2.0 Fluorometer (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). A total amount of 1 µg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext Ultra™ RNA Library Prep Kit for Illumina (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v4-cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina HiSeq 2500 platform and paired-end reads were generated. TopHat2 tools soft were used to map with human genome sequence. Gene expression levels were estimated by FPKM (fragments per kilobase of transcript per million fragments mapped) using Cuffquant and Cuffnorm[27]. Prior to differential gene expression analysis, for each sequenced library, the read counts were adjusted by edgeR program package through one scaling normalized factor. Differential expression analysis of two samples was performed using the DEGseq R package. Pvalue was adjusted using q value. $qvalue < 0.005$ & $|\log_2(\text{foldchange})| \geq 1$ was set as the threshold for significantly differential expression.

Virus infection

Human LV-CON, LV-SFRP1, LV-SFRP1-RNAi, LV-PTGS2 and LV-PTGS2-RNAi were purchased from Genechem (Shanghai, China). Then, the hPTMSCs and A549 cells were infected with LV-CON, LV-SFRP1, LV-SFRP1-RNAi, LV-PTGS2 and LV-PTGS2-RNAi, respectively. SFRP1-targeting small hairpin RNA (shRNA) (ACCTTTTCAGTCCGTGTTTA) and PTGS2-targeting shRNA (TGAATTTAACACCCTCTAT) were cloned into the GV Lentivirus plasmid (Genechem, China). The scramble sequence (TTCTCCGAACGTGTCACGT) of LV-CON was a control, nonspecific shRNA that does not complement any human gene and is not toxic to cultured human cells.

qRT-PCR

Total RNA was isolated using TRIzol Reagent (Ambion, USA). RNA degradation and contamination were monitored on 1% agarose gels. RNA purity and concentration were checked using a Nanodrop™ spectrophotometer (Thermo Fisher Scientific Inc., San Diego, CA, USA). Then, 5 µg of total RNA was converted to cDNA with TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). Afterwards, real-time PCR was carried out using TransStart Tip Green qPCR SuperMix (TransGen Biotech, Beijing, China) on the Bio-rad IQ5 Real Time PCR System (Bio-Rad Inc., USA). The expression levels of gene transcripts were estimated from the respective standard curves, and normalized to the amount of ACTB or GAPDH endogenous control transcripts by the comparative Ct value. Melting curves for each PCR reaction were generated to ensure the purity of the amplification products. Specific primers sets were synthesized by shenggong (Shanghai, China) and illustrated in Table 2.

Table 2
The information of the primers sets used in the quantitative real-time PCR

Name	Gene ID	Forward Sequence(5'-3')	Reverse Sequence(5'-3')	Amplicon Length
C1QTNF1	114897	TGTTACACGGCAAGTTCTAC	ACCTCCTCCTCGTTCTTCAT	115
CD14	929	GCTGGAACAGGTGCCTAAA	CCGTCCAGTGTGAGGTTATC	123
CHI3L1	1116	TGTCGGAGGATGGAACTTTG	ATGGCGGTACTGACTTGATG	92
CXCL16	58191	GCGTCACTGGAAGTTGTTATTG	TGGTAAGCTCTCAGGTGTTTC	100
CXCL8	3576	CTTGGCAGCCTTCCTGATTT	GGGTGGAAAGGTTTGGAGTATG	111
GATA3	2625	GAAGTGTGAGACCAACACAA	GCCTTCCTTCTTCATAGTCAGG	127
GCH1	2643	GATGGTGATTGTGAAGGACATAGA	CCAAGGACTTGCTTGTTAGGA	108
IL18	3606	CAACTCTCTCCTGTGAGAACAA	TTATCATGTCCTGGGACACTTC	118
IRAK3	11213	CGGTCATCTGTGGCAGTATATC	TGCTGCTGCTGGTCATATT	141
KRT18	3875	CCGAGAGGAGCTAGACAAGTA	ATCTCCAAGGACTGGACTGTA	135
MN1	4330	CACTAGTTAAAGCGAGCAGGAG	GGGTCGAGGTAGAGTTAGACAT	141
MTUS1	57509	CCACATTCACAGAGCTCATACA	GCACCATTAAAGGGCATTATC	100
MTUS2	23281	CCTCAGCAAGAGCAGATTCA	CCATACCGAACACGCTAACA	126
PID1	55022	CTCCATCATCTCGACCACAAAG	TGTAGACCCAGGCGAAGAT	121
PLXDC2	84898	GACAGTGCAGCTGAGAAGAA	GCTGATGTTGGGTGGTGATA	128
PRAME	23532	GGTCATGCTGACCGATGTAA	CGTGATCCCACACTCATCAA	100
PTGS2	5743	ATTGACAGTCCACCAACTTACA	CAGGAGGAAGGGCTCTAGTAT	94
PTK2B	2185	GGGAGGTCTATGAAGGTGTCTA	CTTCTCCTTGTTGTCCAGAGTG	101
PTTG1	9232	GGAAGTGTCAACAGAGCTACA	CATCATCTGAGGCAGGAACA	136
SFRP1	6422	GCTTGTGCTGTACCTGAAGA	TCTTGTCCCACTTGTGGATG	134
TACSTD2	4070	CTACTTCGAGAGGGACATCAAG	GGGAATCTCGTCCAGGTAATAG	124
TLR4	7099	GGACCTTTCCAGCAACAAGA	CAGGGACAGGTCTAAAGAGAGA	97
TNFAIP3	7128	CATCCTCAGAAGGCCAATCA	CCAGTGGAGAGGCAAGTAAAT	112
TNFAIP6	7130	ATCTCCAGGCTTCCCAAATG	CAGGTGAATACGCTGACCATAC	85
TNFAIP8	25816	TGAGCTAGCATTGATGGAGAAA	ACACATTCCGGTCAAAGGTATAA	101
TNFAIP8L1	126282	GGATGACACCAGCAGTGAG	GACCAGGTTCTTGAGCATCTT	100
TNFRSF19	55504	TCCTGAACTCACTGGAGAAGA	CAGAATGAGACTGGACTGGAAC	122
TNFSF13B	10673	CTCCAGTCAGAACAGCAGAAA	CTGAGAAGCCATGGAACAAATG	84

Name	Gene ID	Forward Sequence(5'-3')	Reverse Sequence(5'-3')	Amplicon Length
TXNIP	10628	CCAGCCAACTCAAGAGACAA	CAGGAATGAACATGCAGGAAAC	125
LFNG	3955	GACGTCTTCATCGCTGTCAA	ATGAACGTCATCTCCTTGTGG	101
MAML3	55534	ATGGTAGGCTCCCTGAGATTA	TAGGGTCATCGAGAGGTTCTT	111
JAG1	182	GGGAACCCGATCAAGGAAAT	CGCCTCTGAACTCTTACTTCTG	107
SOX2	6657	AGACGCTCATGAAGAAGGATAAG	CCGCTCGCCATGCTATT	76
NANOG	79923	TCCTGAACCTCAGCTACAAAC	GCGTCACACCATTGCTATTC	108
CMYC	4609	CTCCACACATCAGCACAATA	TGTCCAACTTGACCCTCTTG	80
OCT4	5460	ACTATGCACAACGAGAGGATTT	GAGTACAGTGCAGTGAAGTGAG	133
ACTB	60	TCCACGAACTACCTTCAACTC	CAGTGATCTCCTTCTGCATCC	135
GADPH	2597	CTTTGGTATCGTGGAAGGACTC	AGTAGAGGCAGGGATGATGT	133

Western Blot analysis

Whole protein was extracted using Whole Cell Lysis Assay kit and the protein concentrations were detected using BCA Protein Quantification Assay kit (KeyGEN, Nanjing, China). Equal protein from all samples were separated by 10% or 12% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). Membranes were probed with the primary antibodies followed by appropriate secondary antibodies, horseradish peroxidase-conjugated goat anti-rabbit IgG or anti-mouse IgG (BOSTER, Wuhan, China, both at 1:5000). The protein bands were detected via Superstar ECL Plus Ready-to-use (BOSTER, Wuhan, China) and quantified using Gel Imaging System (Bio-Rad Inc., USA). Using β -actin as an internal control, the ratio of grey level between the target bands and the internal control was considered the relative expression of target proteins.

Cell counting kit-8 (CCK-8) cell viability assays

Cells (3×10^3) were seeded onto 96-well plates and incubated in a humidified CO₂ incubator overnight to allow for cell attachment. After all treatments, the CCK-8 assay (Dojindo, Kumamoto, Japan) was performed according to the manufacturer's protocol. The plates were incubated for 1–4 h and then the absorbance was measured at 450 nm in a plate reader (Bio-Rad Laboratories Inc., Hercules, USA). The experiment was performed in triplicates. The following formula was used to determine the percentage of viable cells. Percentage of Cell Viability = OD sample at 450 nm / OD MOCK at 450 nm $\times$ 100%.

Colony-formation assay

The treated cells (1×10^3) were seeded in 6-well plates for colony formation. The cells were incubated in a humidified CO₂ incubator for 10 to 14 days. The colonies were fixed with 4% paraformaldehyde and stained with crystal violet. Colonies of ≥ 50 cells were considered clonogenic survivors.

Wound healing assay

Uniform artificial wounds were made at 3d after infection and the cells were cultured for another 24 h. Cell migration ability was represented by the wound gap distance in microscopic field ($\times 40$) at the time points of 0

and 24 h.

Statistical Analysis

All data were presented with statistical mean $\pm$ standard deviation (SD) from three independent experiments and statistical comparison analysis was performed by T tests using GraphPad Prism software ver.6.0 (GraphPad Software, La Jolla, CA, USA). The significance was set at $p < 0.05$. The comparisons done to obtain statistical significance was between the control (untreated) group and treated groups.

Abbreviations

3-MC, 3-methylcholanthrene; BM-MSCs, bone marrow-derived MSCs; DEGs, differentially expressed genes; FPKM, fragments per kilobase of transcript per million fragments mapped; FDR, False Discovery Rate; hATMSCs, human adipose tissue-derived MSCs; hPTMSCs, human placenta tissue-derived MSCs; MSCs, Mesenchymal stem cells; PAH, Polycyclic aromatic hydrocarbon; PTGS2, prostaglandin-endoperoxide synthase 2; SFRP1, Secreted Frizzled-related protein 1.

Declarations

Ethics approval and consent to participate

The collection of human placentas was approved by the Ethics Committee of Human Research at General Hospital of Ningxia Medical University.

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing Interests

The authors declare that they have no competing interests.

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Authors' contributions

XL and XL conceived and designed the experiments. YJ, XM, XY, JX and TY performed the experiments and acquired the data. YJ, XM and XY analysed the data. YJ drafted the manuscript. XL and XL interpreted the data and critically revised the manuscript. All authors read and approved the final version of the manuscript.

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Figures

Figure 1

Expression levels of differentially expressed genes (DEGs) in DMSO control and 3-MC treated hPTMSCs samples. (A and B) Up-regulated and down-regulated genes are denoted by red and green spots respectively, while not differentially expressed genes are denoted by black spots. (C) A hierarchical cluster analysis of DEGs.

Figure 2

Hierarchical cluster analysis of tumor transformation-related and puripotency-related genes. (A) The hierarchical cluster analysis of tumor transformation-related genes (268). (B) The hierarchical cluster analysis of puripotency-related genes (66). (C) The hierarchical cluster analysis of tumor transformation-related and puripotency-related DEGs (32).

Figure 3

Histogram of GO classification and KEGG pathways of DEGs. (A) The GO classification of DEGs. (B) The percentages of DEGs that were mapped in 50 KEGG pathways of four main KEGG categories. (C) The first 20 KEGG pathways that are enriched with DEGs (Q value ≤ 1.00).

Figure 4

The verification of tumor transformation-related and pluripotency-related DEGs. (A) Detection of mRNA of transformation and pluripotency related DEGs in 3-MC treated hPTMSCs. The mRNA levels was analyzed by qPCR and normalized against GAPDH or ACTB. MOCK was arbitrarily set to 1.0. (B-E) mRNA and protein levels of pluripotent markers SOX2, CMYC, OCT4 and NANOG measured by qPCR and western blotting in hPTMSCs and A549 cells with SFRP1 and PTGS2 overexpression and knockdown.

Figure 5

The inhibitory or promotion effects of SFRP1 and PTGS2 overexpression on the cell viability, colony formation and migration of A549 cells. (A) Detections of mRNA of SFRP1 and PTGS2 overexpression and knockdown efficiencies in stable A549 subcell lines constructed with indicated viruses. (B) Cell viability of A549 cells was analyzed by CCK-8 assay at 24h. SFRP1 silence significantly facilitated the cell viability compared with sh-NC group. PTGS2 silence significantly suppressed the cell viability compared with sh-NC group. (C) Representative images of the indicated A549 subcell lines assessed by colony formation assay. SFRP1 overexpression significantly suppressed the colony formation compared with MOCK or sh-NC group. PTGS2 overexpression significantly facilitated the colony formation compared with MOCK or sh-NC group. (D) Representative images and quantitative data of migration of the indicated A549 subcell lines assessed by wound-healing assay. SFRP1 silence significantly facilitated the migration compared with MOCK or sh-NC group. PTGS2 overexpression significantly facilitated the migration compared with MOCK or sh-NC group. All the experiments were performed in triplicate and the data are presented as the mean $\pm$ SD. * $P \leq 0.05$, ** $P \leq 0.01$ denote significant differences compared with the MOCK or sh-NC group.