Detection of spontaneous tumorigenic transformation during culture expansion of human mesenchymal stromal cells

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Abstract

Human mesenchymal stem/stromal cells (MSCs) have been explored in a number of clinical trials as a possible method of treating various diseases. However, the effect of long-term cell expansion *in vitro* on physiological function and genetic stability is still poorly understood. In this study, MSC cultures derived from bone marrow and liver were evaluated for the presence of aberrant cells following long-term expansion. In 46 independent cultures, four batches of transformed MSCs (TMCs) were found, which were all beyond the culture period of five weeks. These aberrant cells were first identified based on the appearance of abnormal cytology and the acquirement of growth advantage. Despite common MSC markers being diminished or absent, TMCs remain highly susceptible to lysis by allogenic natural killer (NK) cells. When transplanted into immunodeficient mice, TMCs formed sarcoma-like tumors, whereas parental MSCs did not form tumors in mice. Using a combination of high-resolution genome-wide DNA array and short-tandem repeat profiling, we confirmed the origin of TMCs and excluded the possibility of human cell line contamination. Additional genomic duplication and deletions were observed in TMCs, which may be associated with the transformation event. Using gene and microRNA expression arrays, a number of genes were identified that were differentially expressed between TMCs and their normal parental counterparts, which may potentially serve as biomarkers to screen cultures for evidence of early transformation events. In conclusion, the spontaneous transformation of MSCs resulting in tumorigenesis is rare and occurs after relatively long-term (beyond five weeks) culture. However, as an added safety measure, cultures of MSCs can potentially be screened based on a novel gene expression signature.

Keywords: MSCs, tumorigenic, genomic instability, cell culture

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Introduction

Mesenchymal stromal cells (MSCs), originally identified as a heterogeneous population of non-hematopoietic cells in the bone marrow (BM),[1] have the capacity for extensive expansion *in vitro* and differentiate into multiple mesenchymal lineages, including adipocytes, osteoblasts, and chondrocytes.[2,3] In addition to BM, MSCs have been identified in number of postnatal organs where they occupy a perivascular niche.[4] Our group recently demonstrated that the adult human liver harbors a resident cell that is phenotypically and functionally similar to BM-MSCs.[5] Owing to their multilineage potency together with immunomodulatory properties,[6,7] MSCs have attracted widespread interest as a method of treating a number of diseases.

Despite compelling data showing efficacy in preclinical studies using MSCs for the treatment of various diseases in small animals, there is a lack of positive data reported in clinical trials to date.[8] One conceivable explanation could be that insufficient amounts of MSCs are applied in the clinical setting, which may not be a major issue in small animal models. Therefore, optimizing expansion of MSCs in culture may be critical for achieving significant clinical benefit by applying sufficient numbers of MSCs in patients. Extensive expansion of any cell *in vitro* however raises important safety issues, as increasing cell doublings and culture times not only increases the risk of altering the phenotype

of MSCs but also increases the risk of the acquisition of genetic abnormalities.[9,10] Thus, there is an ongoing debate regarding the tumorigenic risk associated with long-term expansion of MSCs *in vitro*.

Murine MSCs have been reported to be susceptible to spontaneous transformation in culture.[11–13] In addition, spontaneous transformation of MSCs derived from cynomolgus macaques[14] has been recently reported. Using short-tandem repeat (STR) analysis, a type of DNA profiling methodology, the authors verified the origin of the transformed cells excluding human cell line contamination.[15] Human MSCs were thought to be genetically stable in culture even following long-term expansion.[16] The first evidence that human adipose-derived MSCs could undergo spontaneous malignant transformation sparked the flame of a scientific controversy.[17] Another independent study subsequently reported that human BM-MSCs frequently undergo spontaneous transformation after long-term culture,[18] whereas an additional study reported that MSCs remain stable after long-term culture.[16] However, these two studies have caused turmoil in the MSCs field, because they later reported that the appearance of transformed malignant cells was due to cross-contamination of human MSCs cultures with a tumor cell line.[19,20] A recent large-scale analysis of various adult stem cells, including MSCs, has recently demonstrated that they can acquire chromosomal aberrations, which may provide a growth advantage.[9] Because human MSCs can acquire chromosomal abnormalities and MSCs have been reported to give rise to sarcomas,[21] there is an urgent need to assess the safety of MSCs following long-term expansion in culture and to better investigate the possibility of spontaneous malignant transformation during cell culture.

The aim of this study was to investigate the occurrence of spontaneous cellular transformation in an independent series of MSCs cultures obtained either from primary human BM or liver. Following long-term expansion of BM- and liver-derived MSCs, four independent cultures out of 46 tested produced transformed cells with tumorigenic potential. High-resolution genome-wide DNA array and STR profiling were used to confirm a shared origin of the transformed cells and parental MSCs. Furthermore, using gene expression analysis and microRNA (miRNA) arrays, we identified a gene expression signature that can be potentially useful for screening for transformation in MSCs cultures.

Materials and methods

Isolation and culture of MSCs

The source of the MSCs is described in supplemental Table S1. Liver MSCs were obtained from explanted livers from patients with end-stage liver disease or from liver graft preservation fluid (perfusates) collected at the time of transplantation. Liver grafts were routinely screened for malignancy. BM-MSCs were either obtained from healthy patients undergoing total hip replacement (Provided by the Department of Otorhinolaryngology, Erasmus MC Rotteradm)[22] or commercially purchased from Lonza (Breda, The Netherlands). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah), 100 IU/mL penicillin, and 100 μg/mL streptomycin. The use of liver tissue, perfusates, and BM was approved by the medical ethical committee of Erasmus MC (Medisch Ethische Toetsings Commissie Erasmus MC). We obtained written informed consent for this original human work.

Flow cytometry

Cells were stained for 30 min at 4°C with directly-labeled mouse monoclonal antibodies directed against CD13-PECy7, CD34-APC, CD45-PERCP, HLA-I-APC (all BD Biosciences), CD73-PE, CD166-PE (BD Pharma, San Jose, CA), and CD105-FITC (R&D Systems, Abingdon, UK). Flow cytometric analysis was performed using the FACSCanto II (BD Biosciences) and 10,000 events were collected for analysis performed using FlowJo software.

Gene and miRNA expression profiling by microarray

The total RNA (including small RNA) from parental MSCs and transformed MSCs (TMSCs) cultures was isolated using a Qiagen miRNeasy Mini Kit (Qiagen, Venlo, The Netherlands) and used for genome-wide gene expression analysis with the HuGene 1.0 ST Genechip or miRNA Array (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions. Transcript levels were generated using RNA as implemented in the Affymetrix Gene Expression Console and probesets annotations were retrieved from Nettafx using the same software. Probesets differentially expressed among conditions were analyzed using Partek Genomics Suite (Partek Incorporated, MO). Pathway analysis was performed using Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA).

High-resolution genome-wide DNA analysis

Genomic DNA of parental and TMSCs was isolated using QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. High-resolution genome DNA profiling was performed using the Affymetrix® Cytogenetics Whole-Genome 2.7 M Array (Affymetrix, Inc.). This array provides unbiased, whole-genome coverage with the highest density of 2.7 million markers to enable superior resolution of detecting chromosome aberrations. Data analysis was performed with Affymatrix Chromosome Analysis Suite (Affymetrix, Inc.) that contains a build-in human genome as a reference. To generate a karyoview of TMCs and MSCs, a 750 kB cutoff was used to detect genome loss or gain variations.

STR profiling

The parental and TMSCs were genotyped by STR profiling using the PowerPlex 16 System (Promega), as previously described.[23] The PowerPlex 16 system is composed of 15 STR loci. Amplification was done using 1 ng of template DNA, which was applied to the PowerPlex 16 system following the manufacturer's recommendations. Multiplex polymerase chain reaction (PCR) reactions were carried

out using fluorescent dye-linked primers. Labeled products were detected by electrophoretic size fractionation on an ABI 3100 genetic analyzer. The data were analyzed using Genescan and Genotyper software (Perkin-Elmer) to categorize peaks according to their size in relation to an internal standard run. This analysis enabled every peak to be allocated a size corresponding to the number of repeat units present.

Real time (RT)-quantitative PCR (RT-qPCR)

Total RNA was isolated by a Qiagen miRNeasy Mini Kit and quantified using a Nanodrop ND-1000 (Wilmington, DE). For gene expression, cDNA was prepared from the total RNA using an iScript cDNA Synthesis Kit from Bio-Rad (Bio-Rad Laboratories, Stanford, CA). Gene expression was quantified using real-time PCR (MJ Research Opticon, Hercules, CA) using SybrGreen (Sigma-Aldrich) according to manufacturer's instructions. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene to normalize gene expression. For miRNA expression, cDNA was prepared by a TaqMan miRNA reverse transcription kit (Applied Biosystems, Carlsbad, CA). RT-qPCR reactions were performed using a TaqMan Universal PCR Master Mix and miRNA-specific PCR primers (Applied Biosystems) according to the manufacturer's instructions. A small nuclear RNA, RNU43, served as a reference gene.

MTT assay

Parental MSCs or TMCs (5×10^3 cells) were plated in 96-well plates. At the indicated times, the number of metabolically active cells was quantified by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, 0.5 mg/mL) assay.

Cytotoxicity-mediated lysis by europium release assay

Cytotoxicity-mediated lysis of target cells was determined by europium release assays as previously described.[24] For effector cells, peripheral blood mononuclear cells (PBMCs) derived from healthy volunteers were stimulated with allogeneic γ-irradiated PBMCs and 200 IU/mL IL-2 (proleukin; Chiron BV, Amsterdam, The Netherlands) in round-bottom 96-well plates for 7 days at 37°C. MSCs were used as target cells.

Maximum release of europium by target cells was measured by incubation of 5×10^3 labeled target cells with 1% triton (Sigma-Aldrich, Zwijndrecht, The Netherlands) for 4 h. Spontaneous release of europium by target cells was measured by incubation of labeled target cells without effector cells. The percentage of leakage was then calculated as: (spontaneous release/maximum release) × 100%. Finally, the percentage of cytotoxicity-mediated lysis was calculated as: % lysis = [(measured lysis − spontaneous release)/(maximum release − spontaneous release)] × 100%.

Engraftment of cells into immunodeficient mice

Immunodeficient NOD/SCID mice (Erasmus MC institutional breeding) were maintained in the Erasmus MC animal facility on a 12/12 h light/dark schedule. The animals had free access to food and drinking water. When reaching the age of 6–8 weeks, the mice were subcutaneously injected with 1×10^6 parental MSCs or TMCs (two independent batches into four mice) labeled with or without luciferase reporter gene, as previously described.[5] Luciferase activity was measured at the indicated time points by an *in vivo* imaging system (IVIS) camera in

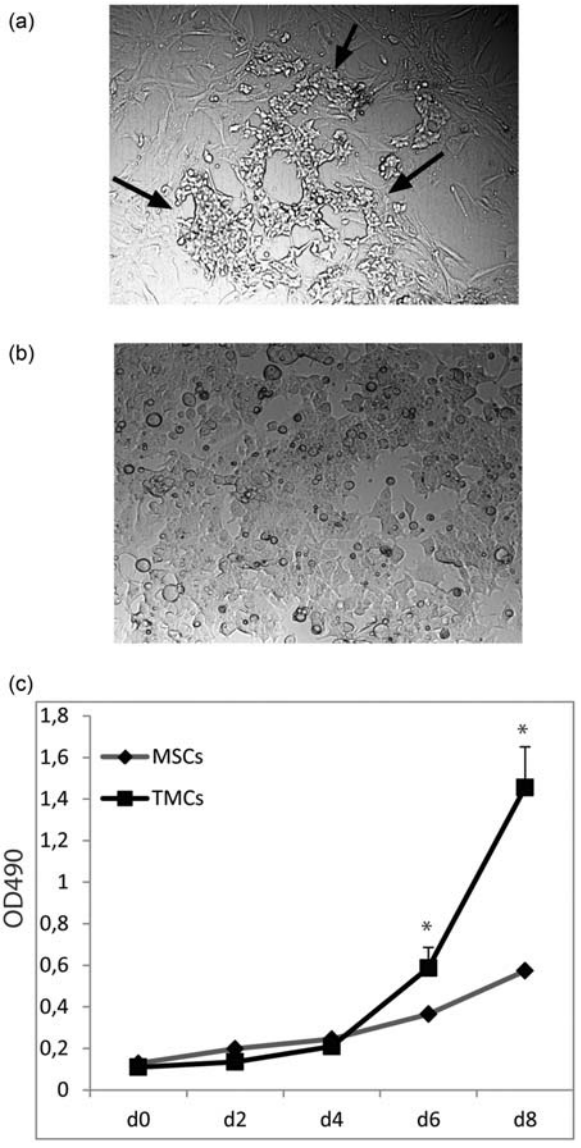


Figure 1 Spontaneous transformation of human MSCs during culture. (a) A representative image of a cluster of spontaneously transformed cells with epithelial-like morphology emerged in MSCs cultures (a batch of BM-MSCs represented), which clearly differ from the typical fibroblast-like morphology of normal MSCs (indicated by arrow). (b) These cells can further expand in culture. (c) An MTT assay showed the accelerated proliferation pace of transformed MSCs, compared with normal MSCs. At day 6 and 8, the OD490 values of TMCs are significantly higher than that of MSCs ($n = 6$, $^*P < 0.01$). BM: bone marrow; MSCs: mesenchymal stem cells; MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; TMC: transformed mesenchymal stem cell

living animals. One pair of luciferase-labeled TMCs and MSCs were injected at different sites in the same recipient NOD/SCID mouse.

To avoid discomfort caused by the over-growing tumor, mice were sacrificed after 4 weeks by CO_2 inhalation. The tumors were partially resected for histology evaluation and partially dissociated for culturing tumor cells. The use of animals was approved by the institutional animal ethic committee (Dier Experimenten Commissie).

Statistical analysis

Statistical analysis was performed by using either the paired nonparametric Wilcoxon signed-rank test, or the unpaired nonparametric Mann–Whitney test, using GraphPad InStat software (GraphPad Software Inc., San Diego, CA). P values <0.05 were considered statistically significant.

Results

Spontaneous transformation of human BM- and liver-derived MSCs after long-term culture expansion

MSCs cultures were obtained from human BM ($n=5$), liver biopsies ($n=12$), or liver graft preservation fluid ($n=29$). Donor and culture characteristics are shown in supplementary Table S1. In four independent MSCs cultures beyond five weeks of *in vitro* expansion (Table S1), we observed aberrant looking cells (transformation) with an epithelial-like cytology, as shown in Figure 1. Initially, these aberrant MSCs appeared as a few scattered colonies (Figure 1a) but gradually these cells acquired a growth advantage and overgrew the normal appearing MSCs in the culture (Figure 1b). Quantification of the viable cells confirmed the accelerated proliferation of the aberrant MSCs compared to parental MSCs cultures (Figure 1c). These aberrant MSCs could be expanded in serum-containing medium for at least 30 passages over a three-month period without any evidence of senescence (data not shown). For liver-derived MSCs, derived from either preservation fluid or tissue biopsies, we observed two aberrant cells in 41 independent cultures (Table S1). For BM-derived MSCs cultures, we observed two positive cultures out of five (Table S1). Importantly, all samples were obtained from donors with no evidence of pre-existing malignancy.

Aberrant MSCs have tumorigenic potential

We next investigated the tumorigenic potential of the aberrant MSCs. For this, 1.0×10^6 unlabeled aberrant MSCs or MSCs labeled with a luciferase reporter gene were subcutaneously injected in NOD/SCID mice. Within a period of four weeks, both unlabeled and luciferase-labeled aberrant MSCs formed solid tumors following subcutaneous injection ($n=4$; Figure 2a). The formation of solid tumors under the skin was visible as early as two weeks after injection. Due to symptoms related to tumor burden, mice were sacrificed 4–5 weeks post-injection and the tumors were harvested. Histological examination of the tumor tissue revealed a sarcoma-like pathology (Figure 2b).

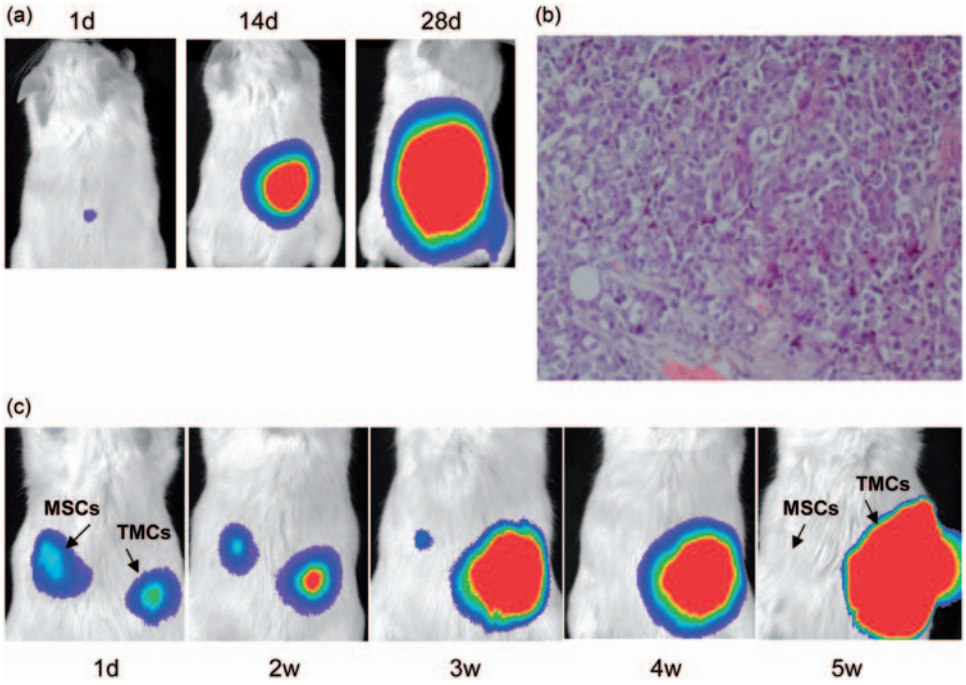


Figure 2 Transformed but not parental MSCs are tumorigenic in immunodeficient mice. (a) BM or liver TMCs were labeled with a lentiviral expressed luciferase gene and subcutaneously injected into immunodeficient NOD/SCID mice ($n=4$). Luciferase activity was longitudinally monitored by IVIS camera in living mice at indicated time points. (b) H&E-stained tumor sections showing a liposarcoma-like histology. (c) Simultaneous engraftment of one pair of luciferase-labeled TMCs and MSCs into the right and left lower right flanks of NOD/SCID mice, respectively. The luciferase signal from TMCs dramatically increased, whereas the signal from MSCs decreased over time. BM: bone marrow; H&E: hematoxylin and eosin; IVIS: in vivo imaging system; MSCs: mesenchymal stem cells; TMC: transformed mesenchymal stem cell. (A color version of this figure is available in the online journal)

To confirm the tumorigenicity gained was due to spontaneous transformation, we simultaneously injected luciferase-labeled TMCs and normal MSCs derived from the same origin at different sites in the recipient NOD/SCID mouse. As shown in Figure 2c, the luciferase signal from TMCs dramatically increased with time, resulting in the formation of palpable tumors. In contrast, the signal from the normal parental MSCs diminished over time and by 4 weeks was no longer visible, which is in agreement with our previous data[5] showing no tumor formation after engraftment of both BM- or liver-derived MSCs either subcutaneous or intrahepatic ($n = 14$). Following dissociation of the explanted tumor tissue, viable cells were cultured, which were termed as T-TMCs. These cells demonstrated a similar cytology and proliferation capacity as primary TMCs prior to injection and could be expanded for at least five passages as tested so far (Figure S1). These data confirmed the tumorigenicity and malignancy status of these transformed cells.

Common MSCs markers are diminished in TMCs, and they remain susceptible to cell lysis by natural killer cells

We next compared the antigenic profile of TMCs to parental MSCs using flow cytometry. Common MSCs markers CD13, CD73, CD105, and CD166 were down-regulated in TMCs compared to their normal parental counterparts (Figure 3). However, the common hematopoietic markers, CD34 and CD45, remained negative. In addition, we also showed that T-TMCs harvested from tumors grown in NOD/SCID mice and expanded *in vitro* possess a similar antigenic profile to the TMCs, which was unexpected (Figure 3).

It is known that MSCs are susceptible to rapid killing by natural killer (NK) cells, which is presumed to contribute to the destruction of allogenic MSCs after transplantation into an immune competent host.[25,26] We next investigated whether TMSCs remain susceptible for lysis by NK cells. As shown in Supplementary Figure S2, TMCs were highly susceptible to lysis by activated NK cells. In addition, T-TMCs cultured from explanted tumor tissue from NOD/SCID mice were lysed by NK cells, comparable to MSCs and TMCs (Figure S2).

STR analysis authenticates the origin of the TMSCs excluding the possibility of human cell line contamination

To address the identity of the TMCs, we used DNA profiling and STR analysis. Genomic DNA from both TMCs and parental MSCs were subjected to high-resolution genome DNA profiling by using the Affymetrix® Cytogenetics Whole-Genome 2.7 M Array. As shown in Figure 4a, an identical signature in copy number variation (CNV) was observed between one pair of TMCs and MSCs, providing evidence that both cell types were of the same origin. Of note, these common CNVs were either inherited or gained *de novo* during the culture of parental MSCs before transformation. In contrast, the karyoview of two individuals, generated from genomic DNA of PBMCs and analyzed by 750 kB cutoff as well, displayed distinct CNV patterns (Figure S3).

To authenticate the origin of the TMCs, we profiled 15 STR loci and one sex chromosome marker Amelogenin from two independent pairs of TMCs (Figure 4b). Based on the STR analysis, parental and TMSCs have an identical STR signature (Figure 4b). We also compared the data obtained from the STR profiling to the ATCC STR database

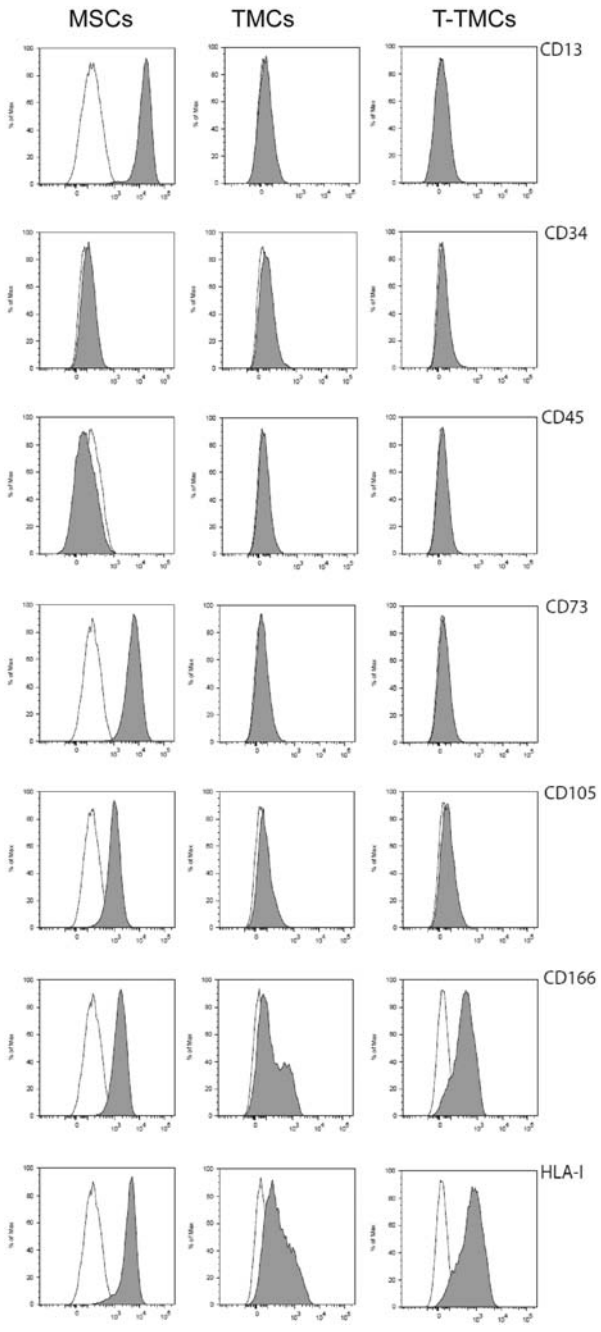
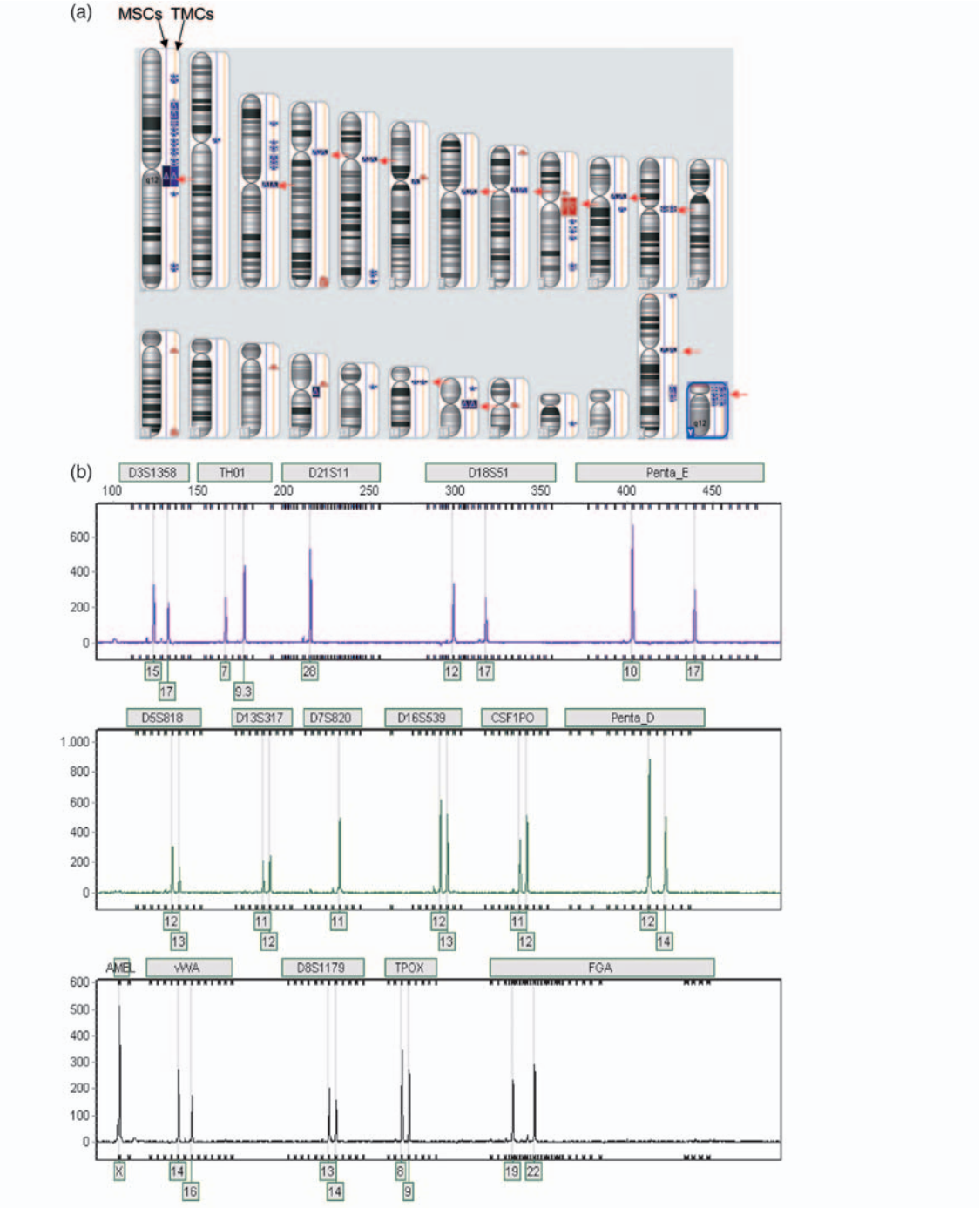


Figure 3 Transformed MSCs loose mesenchymal markers. Flow cytometric analysis revealed the down-regulation of the common MSC markers CD13, CD73, CD105, and CD166, whereas the hematopoietic markers, CD34 and CD45, remained negative. MSCs, normal MSCs; TMCs, transformed MSCs and T-TMCs, tumor cells isolated from TMCs-formed solid tumors in mice. This figure showed one representative transformed BM-MSCs batch. MSCs: mesenchymal stem cells



	Penta_E	D18S51	D21S11	TH01	D3S1358	FGA	TPOX	D8S1179	vWA	Penta_D	CSF1PO	D16S539	D7S820	D13S317	D5S818	Amg
MSC-1	10, 17	12, 17	28	7, 9.3	15, 17	19, 22	8, 9	13, 14	14, 16	12, 14	11, 12	12, 13	11	11, 12	12, 13	X
MSCT-1	10, 17	12, 17	28	7, 9.3	15, 17	19, 22	8, 9	13, 14	14, 16	12, 14	11, 12	12, 13	11	11, 12	12, 13	X
MSC-2	7, 15	17, 18	28, 30.2	7, 9.3	15, 16, 17	23	10, 1	1 11, 12, 14	17, 19, 20	9, 10	11, 12	9, 13	10, 11	12	8, 9	X
MSCT-2	7, 15	17, 18	28, 30.2	7, 9.3	15, 16, 17	23	10, 1	1 11, 12, 14	17, 19, 20	9, 10	11, 12	9, 13	10, 11	12	8, 9	X

Figure 4 Genomic DNA comparison of parental and transformed MSCs. (a) High-resolution Affymetrix® Cytogenetics Whole-Genome 2.7 M Array was used to map the genomic DNA. To generate a clear karyoview of transformed MSCs (TMCs) and parental MSCs, a 750 kB cutoff was used to detect genome loss (red) or gain variations (blue) as referred to the human genome built in the Affymatrix Chromosome Analysis Suite software. An identical signature (arrow indicated) of CNV was observed between TMCs and MSCs, supporting the same origin of the cells. These common CNVs were either inherited or gained *de novo* during the culture of parental MSCs before transformation. (b) STR profiling of transformed and parental MSCs. STR loci are indicated in boxes above the electropherogram; numbers of repeat units are indicated below the peaks. Two pairs of transformed and parental MSC showed identical profiles, suggesting that *de novo* transformation, not cell-contamination, occurred. CNV: copy number variation; MSCs: mesenchymal stem cells; STR: short-tandem repeat. (A color version of this figure is available in the online journal)

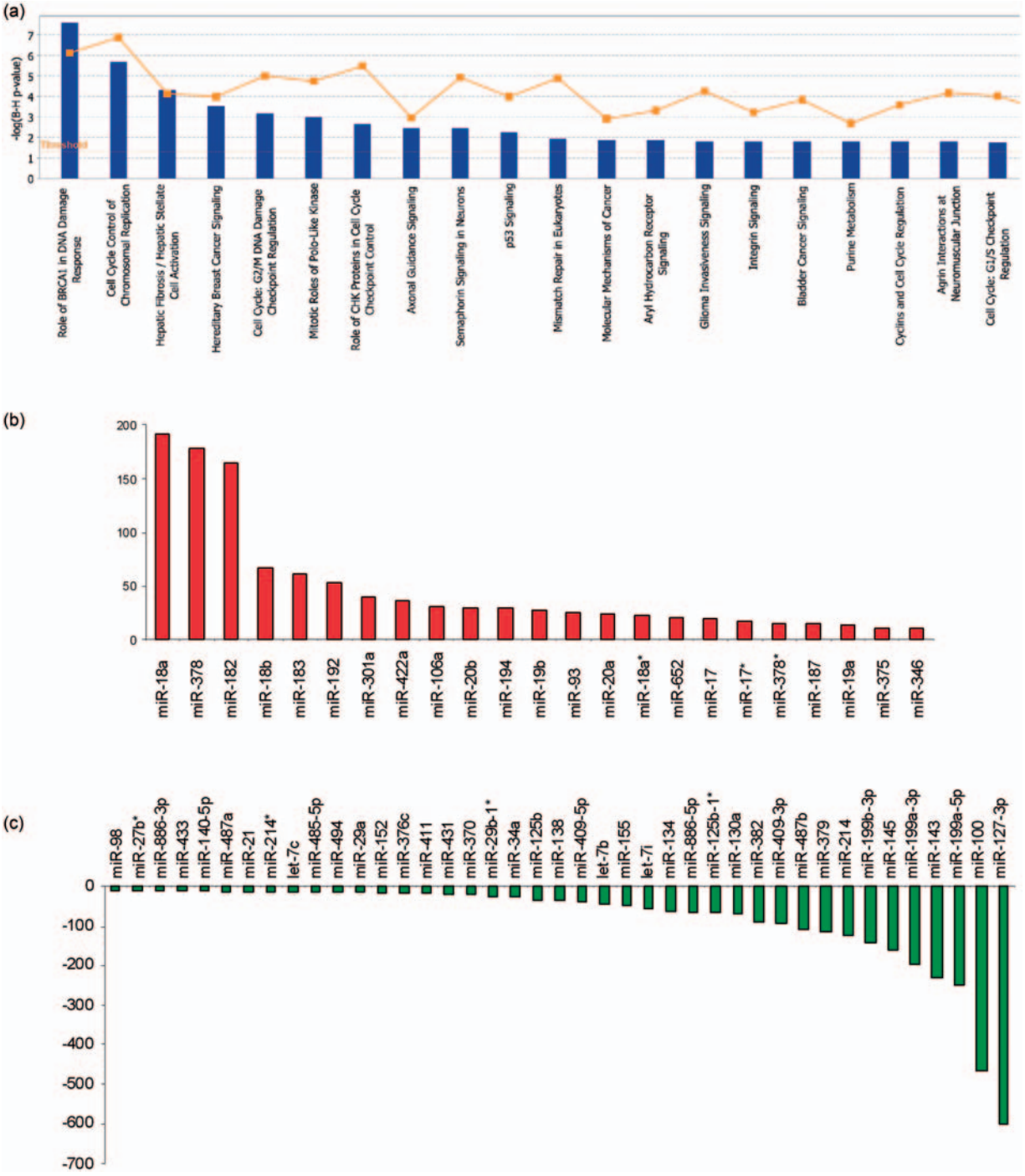


Figure 5 Gene/miRNA expression profiling and pathway analysis. (a) The expression of 3598 genes (28,870 probe sets in total) were found to be up- or down-regulated more than two-fold in a paired TMCs versus MSCs. Ingenuity Pathway Analysis identified 31 canonical pathways that were significantly enriched for these genes (B-H multiple testing correction, $P < 0.05$), which were associated with cell cycling or cancer signaling. Genome-wide profiling of 853 human miRNAs identified 23 miRNAs that were up-regulated more than 10-fold (b) and 41 were down-regulated over 10-fold (c) in TMCs compared to parental MSCs. MSCs: mesenchymal stem cells; TMC: transformed mesenchymal stem cell. (A color version of this figure is available in the online journal)

and no hits were obtained, further excluding the possibility of human cell line contamination.

Further analysis of the cytogenetics array showed additional unique CNVs present in TMCs (Figure S4). In particular, chromosome 1 abnormalities are present in TMCs compared with parental MSCs (Figure 4a and Figure S4). Chromosome 1 abnormalities are a frequent occurrence in cancer,[27] however, the role that this plays in contributing to the growth advantage and tumorigenic potential in our study requires further clarification.

TMCs demonstrate unique global gene and miRNA expression changes

We next characterized the genomic features of the TMCs by performing genome-wide gene expression and miRNA

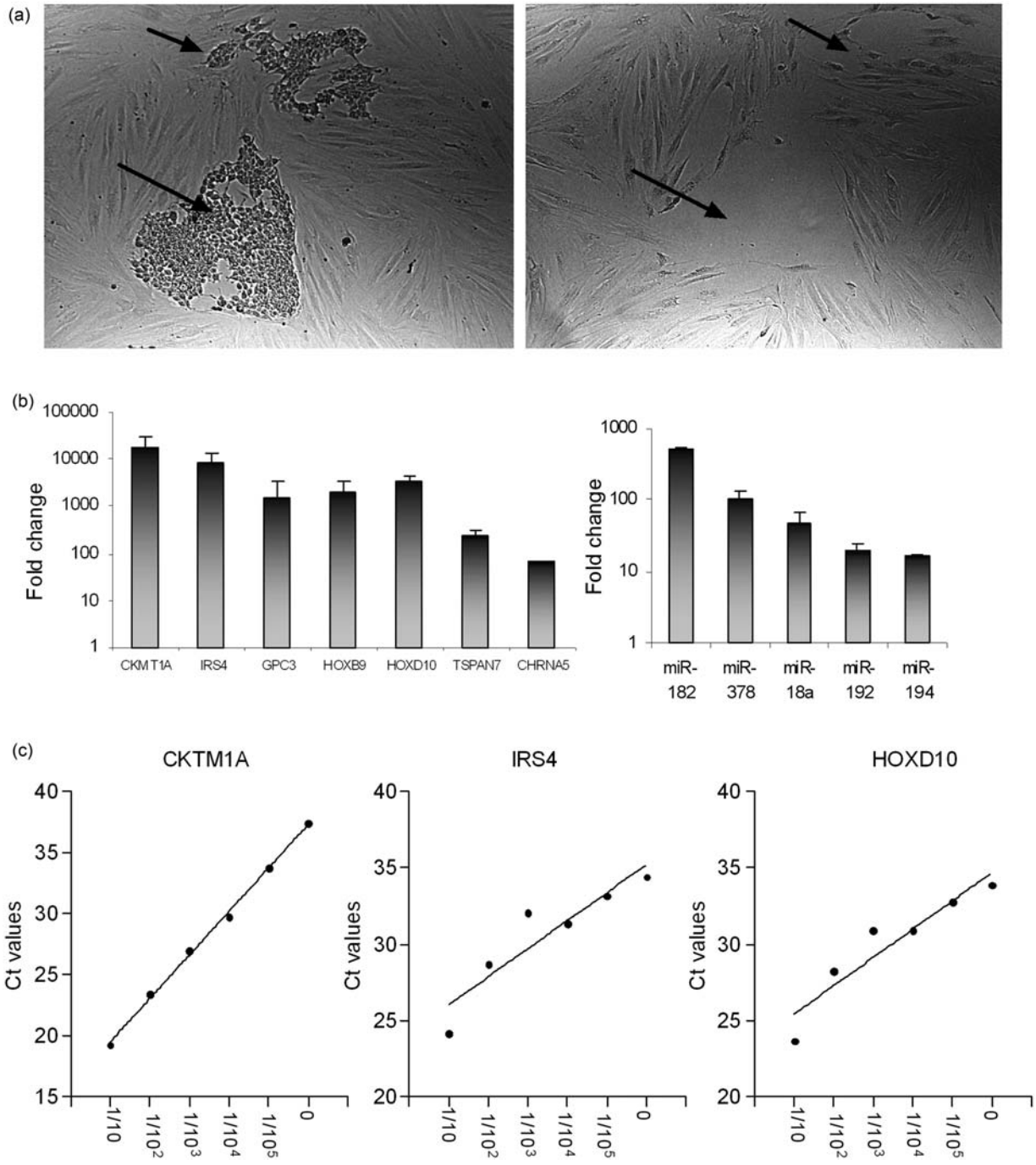


Figure 6 Screening transformed MSCs using biomarkers. (a) Transformed MSCs (arrow, a batch of BM-MSC represented) were observed as low plastic adhering cells, which can be mechanically detached without affecting normal MSCs. A set of genes (b) and miRNAs (c) were found to be dramatically up-regulated in transformed MSCs. (d) TMCs and MSCs were cultured at indicated ratios. After mechanical pipetting to dissociate low adhering cells, the supernatant was collected and the cells were harvested by centrifugation. QRT-PCR was carried out using CKTM1A, IRS4, and HOXD10 to detect transformed MSCs. The X-axis indicates the *Ct* value of qRT-PCR and the Y-axis indicates the ratio of mixed to transformed MSCs. The data represent one of the three independent experiments. BM: bone marrow; MSCs: mesenchymal stem cells; qRT-PCR: quantitative real time polymerase chain reaction; TMC: transformed mesenchymal stem cell

arrays using total RNA isolated from TMCs and parental MSCs. The expression of 3598 genes (28,870 probe sets in total) were found to be up or down-regulated more than two-fold in TMCs versus MSCs. The global function of these differentially expressed genes was subjected to a functional and pathway analysis using Ingenuity Pathway Analysis software, which identified 31 canonical pathways that were significantly altered between MSCs and TMCs (B-H multiple testing correction $P < 0.05$; Figure 5a). Many of these pathways were associated with cell cycling or cancer signaling.

Genome-wide profiling identified 23 miRNAs that were up-regulated more than 10-fold (Figure 5b) and 41 that were down-regulated over 10-fold (Figure 5c) in TMCs compared to parental MSCs. Overall, a greater number of miRNAs were down-regulated in TMCs, which is consistent with a

previous report describing a general down-regulation of miRNAs in various cancers.[28] In particular, the let-7 family, comprised of 12 family members (let-7-a1, a2, a3, b, c, d, e, f1, f2, g, i and miR-98), have been described as major guardians against pluripotency and cancer progression, and are often found to be down-regulated during cancer progression.[29] Four of these members were down-regulated over 10-fold in TMCs, namely let-7i (58-fold), let-7 b (43-fold), let-7 c (14-fold) and miR-98 (11-fold) (Figure 5c). Notably, among the most highly up-regulated miRNAs in TMCs (Figure 5b), miR-378 (178-fold) is known to promote cell survival, tumor growth and angiogenesis[30] and miR-183 (61-fold) has oncogene function and been shown to be over-expressed in tumor tissues.[31]miRNA-199a-3p was down-regulated in TMCs by 195-fold. This is consistent with a recent study that miRNA-199a-3p is down-regulated in human osteosarcoma and regulates cell proliferation and migration.[32]

To integrative analyze the gene and miRNA regulation, we combined the 2-fold differentially expressed gene list with 10-fold differentially expressed miRNA list and then subjected them to Ingenuity Pathway Analysis. Within the 25 networks analyzed, there are 7 networks composed of the interaction of miRNAs with genes, including the top second, third and fourth scored networks (Figure S5a). The functions of these most significant networks are associated with cancer (Figure S5b), migration (Figure S5c), and amino acid metabolism (Figure S5d). Taken together, the integrative molecular mapping reflects the transformed status of the MSCs.

Screening for spontaneous transformation using a gene and miRNA expression signature

Plastic adherence is a defining feature of MSCs.[33] However, we observed that aberrant cells readily detached from the plastic surface upon applying shear stress in each independent culture. As shown in Figure 6a, applying shear stress resulted in the complete detachment of TMCs cells without affecting the attachment of the normal MSCs present in the same culture flask. Since the culture of clinical grade MSCs often use adherent plastic flasks,[34] potentially transformed cells can be removed by mechanically detaching low adhesive cells and these cells can be harvested by centrifugation of the discarded culture medium.

We next selected a set of genes and miRNAs, which we had found to be highly expressed in TMCs (Figure 5) and confirmed their relative expression in three independent pairs of MSCs and TMCs using qRT-PCR. As shown in Figure 6b, *CKMT1A* was elevated over 10,000-fold, and several other genes were also elevated over a 1000-fold in TMCs. Similarly, miR-182 and miR-378 were elevated approximately 500- and 100-fold, respectively (Figure 6c). To determine the sensitivity of detection of TMCs, we performed mixed culture experiments with different ratios of TMCs and MSCs. After mechanical pipetting to dissociate low adhering cells, the supernatant was collected and the cells were harvested. qRT-PCR was performed on the following genes: *CKTM1A*, *IRS4* and *HOXD10*, to detect TMSCs (Figure 6d), where transformed cells could be identified at a resolution of 1:10,000 by screening the collected low adhesion cells using this panel of genes and miRNAs by quantitative RT-PCR.

Discussion

The study of transformation of mammalian cells in culture has flourished over decades. Virus-induced, carcinogen-induced or spontaneous transformation has been investigated mainly for the purpose of serving as an assay for titrating viruses, evaluating specific carcinogens or studying the mechanisms of carcinogenesis.[35] The first report of the spontaneous malignant transformation of cultured primary cells was published in 1941, where transformed rat fibroblasts in culture were able to form sarcomas when transplanted into rats.[36] Meanwhile, another study reported that transformed mouse fibroblasts also gave rise to sarcomas after injection into the mice of the inbred strain of origin.[37]

In the 1950s, it was discovered that the BM harbors mesenchymal stem/stromal cells, which have a typical fibroblast-like morphology. More recent studies report the presence of MSCs in other compartments including adult adipose tissue, dermal tissues, spleen, liver as well as umbilical cord blood and various fetal tissues. Since the therapeutic application of MSCs requires an extensive expansion of the cells in culture, the study of tumorigenic transformation of MSCs is an important clinical issue. Spontaneous transformation of murine and monkey MSCs has previously been observed,[11–15] and the accumulation of chromosomal instability as a result of expansion in culture has been implicated as the potential mechanistic cause.[11] Although human MSCs are genetically more stable compared with murine MSCs, genomic instability has also been reported in cultured human MSCs.[38] However, controversy regarding the transformation of MSCs remains, as two recent reports that initially described MSC transformation following cell culture were retracted due to the discovery of contamination of the primary cell cultures with tumor cell lines,[19,20] In our study, we observed the spontaneous transformation of human liver- and BM-derived MSCs after long-term (more than five weeks) expansion *in vitro* with tumorigenic consequences. The epithelial-like cells in MSCs cultures acquired a growth advantage and eventually overgrew the normal MSCs. These transformed cells formed sarcoma-like tumors after injection into NOD/SCID mice, supporting their tumorigenicity *in vivo*. We authenticated the origin of the TMSCs using both high-resolution genome-wide DNA analysis and STR profiling, a standard technique for characterizing cell identify,[39] and our results demonstrate that the TMSCs originated from the original MSCs donors, but not due to tumor cell contamination as previously described.[19,20] Further characterization of TMSCs by genome-wide gene and miRNA expression with integrated pathway analysis confirmed their tumorigenic status. Interestingly, solid evidence based on transgenic mice and the genetic investigation of MSCs has placed these cells as the most likely cell of origin for certain sarcomas.[21] In addition, a clinical study reported the development of osteosarcoma in a recipient 17 years

after BM transplantation. They further demonstrated the osteosarcoma cell line established from the recipient's tumor carried a marker of chimerism and expressed a panel of MSCs markers, suggesting its origin as being from donor MSCs.[40]

The therapeutic potential of adult stem cell therapies, including MSCs, is promising due to the growing need for new medicines to treat a number of debilitating diseases, which currently lack adequate treatments.[41–43] However, there is a growing need to develop novel methods to assess the safety of expanded adult stem cells. One of the major concerns, yet still controversial, is the tumorigenic potential of adult stem cell cultures, including MSCs. Although limited in their differentiation potential, there is increasing evidence that MSCs do contribute, in part, to the growth and spread of solid tumors. However, one of the limiting factors in the MSCs field is bioequivalence. It is still not known whether BM-MSCs, which represent the main cell type used in the human clinical trials to date, and stromal cells found in the tumor microenvironment are bioequivalent. Second, and even more controversial, is whether MSCs themselves are tumorigenic, such as has been suggested for certain soft tissue sarcomas. Clearly, the debate still continues and needs to be clarified.

One of the defining features of human MSCs is their dependence on anchorage, allowing their adherence to plastic surfaces. We noted that in the MSCs cultures, aberrant cells had low adhesive properties. TMSCs appeared after long-term culture, indicating that early passage cultures are likely safe to use. Furthermore, we have identified a panel of highly up-regulated miRNAs and genes in TMSCs using genome-wide miRNA and gene expression arrays, which could be used as potential biomarkers to screen for transformed cells during culture. In fact, the establishment of safety evaluation makers is one of top priorities in the stem cell field.[44] Using the low adhesive properties of TMSCs, the sensitivity of screening assays could be dramatically increased by harvesting only the low adhesive cells. Obviously, heterogeneity probably exits that not all the transformed cells express the same markers. Thus, the utility of this approach still require further investigation and validation.

In summary, we observed the tumorigenic transformation of human MSCs during long-term culture expansion, which was not due to contamination of human cell lines. Furthermore, we identified a number of genes and miRNAs using gene arrays and qRT-PCR that may potentially be used to screen for transformation events in long-term cell culture. This approach would be a significant step towards the clinical application of stem cell therapy, and would help to alleviate the concern of transplanting malignant cells into patients.

Author contributions: QP designed and performed the experiments, analyzed the data, and wrote the manuscript; SMGF, PER, and WNMD performed experiments and analysis; JK and HWT provided (clinical) input/samples and edited the manuscript and LJWL designed and supervised the research, analyzed the data, and edited the manuscript.

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