The Impact of Cell Source, Culture Methodology, Culture Location, and Individual Donors on Gene Expression Profiles of Bone Marrow-Derived and Adipose-Derived Stromal Cells

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Bone marrow (BM) stromal cells (MSCs), also known as mesenchymal stem cells, display a high degree of heterogeneity. To shed light on the causes of this heterogeneity, MSCs were collected from either human BM ($n=5$) or adipose tissue (AT) ($n=5$), and expanded using 2 different culture methods: one based on fetal calf serum, and one based on human platelet lysate. After initial expansion, MSCs were frozen, and the vials were transported to 3 different laboratories and grown for 1 passage using the same brand of culture plastic, medium, and supplements. Subsequently, the cells were harvested and assayed for their gene expression profile using the Affymetrix exon microarray platform. Based on gene expression profiles, the most discriminative feature was the anatomical harvesting site, followed by culture methodology. Remarkably, genes in the WNT pathway were expressed at higher levels in BM-derived MSCs than in AT-derived MSCs. Although differences were found between laboratories, cell culture location only slightly affects heterogeneity. Furthermore, individual donors contributed marginally to the observed differences in transcriptomes. Finally, BM-derived MSCs displayed the highest level of similarity, irrespective their culture conditions, when compared to AT-derived cells.

Introduction

WHEN BONE MARROW (BM) stromal cells (MSCs) were first recognized [1], it was soon realized that those cells showed potential for the repair of tissues such as bone and cartilage [2,3]. Based on their immunosuppressive function, they were considered ideal donor cells to repair heart tissue affected by myocardial infarction or ischemic brain injury [4–8]. The differences in differentiation abilities that were observed by different laboratories were initially assigned to the use of different sources and culture conditions of MSCs [9]. However, several reports described functional as well as phenotypic heterogeneity of MSCs, which is also reflected by their diverse nomenclature: mesenchymal stem cells, bone MSCs, or multipotent stromal cells [10–19]. Also, *in vivo* and *in vitro* differences in differentiation add to the confusion [20]. Here, we will call them MSCs, which can be read as MSCs, multipotent stromal cells, or mesenchymal stem cells. The

source of the cells either BM or adipose tissue (AT) will be indicated upfront of MSCs. Adding to the degree of heterogeneity is the anatomical location of harvest sites for these cells [21–26]. Since AT can be obtained with low-invasive procedures and donors tend to easily donate AT, this source of MSCs became popular in contrast to BM aspiration [27]. Notwithstanding the higher frequency of MSCs in AT tissue than in BM [28,29], the frequency of MSCs is still rather low in both tissues, and an expansion step is needed to obtain enough cells for experimentation, which further leads to increased heterogeneity [29–31]. Moreover, the phenotypic and functional heterogeneity is further increased by the use of different expansion protocols and reagents. Selected batches of fetal calf serum (FCS) are in use, based on the property that MSCs should expand while maintaining their multipotency. Those serum batches are similar due to extensive analysis of the manufacturer, but they are not equivalent. A further cause of heterogeneity is the use of human platelet lysate (hPL) for

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expansion of MSCs [25,32–36]. Expansion in hPL is attractive because it does not contain animal components [32,35,37], which allows cells cultured in hPL to be used in clinical settings [38]. However, the culture of MSCs in highly selected batches of FCS for clinical purposes is allowed. Significant differences in the cell cycling time have been reported when culturing in hPL compared to FCS [39,40]. Conforming to their definition, all MSCs should be able to differentiate into osteoblasts, chondrocytes, and adipocytes, irrespective their culture history and cell source. However, marked differences were observed in the phenotype and differentiation capacity of MSCs [41,42], as well as their immunosuppressive capacity [43]. This observed heterogeneity was at least partly ascribed to donor-to-donor variation.

To clarify the cause of the differences observed in the functionality and phenotype, we performed experiments to unravel the sources that influence the reported heterogeneity. We isolated MSCs from BM ($n=5$) as well as AT ($n=5$) to deduce the importance of the tissue source. Moreover, we cultured the cells using FCS and hPL to investigate the effects of different medium supplements. Finally, after the initial expansion phase in either FCS or hPL performed at one location, the same cells were cultured in 3 different laboratories. After cell culture, gene expression profiles were generated and analyzed using the Affymetrix exon array platform. This study provides an important step in the clarification of the impact of cell source, culture methodology, different culture laboratories, and individual donors on the gene expression profiles of MSCs, and thereby the potential causes of heterogeneity in MSC cultures.

Materials and Methods

Collection and initial expansion of the MSCs

BM was obtained from 5 donors after signing written informed consent. The gender and age at the moment of donation were as follows: F, 46; M, 39; F, 33; F, 49; and M, 29. AT was obtained from 5 patients undergoing orthopedic surgery, also after signing written informed consent. The gender and age for these donors were as follows: F, 8; M, 5; M, 2; F, 8; and F, 2. The samples were processed and expanded in hPL as well as FCS. BM mononuclear cells were isolated by Ficoll density-gradient centrifugation and plated in polystyrene culture flasks at a density of $160,000/\text{cm}^2$ in a complete culture medium (low glucose [LG]-DMEM; Invitrogen, Paisley, United Kingdom) supplemented with penicillin and streptomycin (Lonza, Verviers, Belgium) and 10% preselected FCS (HyClone, Logan, UT). Cells were collected by trypsinization at 90% confluency. To obtain AT-derived MSCs, leftover subcutaneous or retroperitoneal fat tissue was obtained. The fat was minced, placed in phosphate-buffered saline (PBS) containing 0.4% human serum albumin, treated with liberase H1 (Roche, Indianapolis, IN) at 37°C for 60 min, under frequent shaking, and subsequently centrifuged for 10 min at 500 g at room temperature. The supernatant, containing mature adipocytes, was aspirated, and the stromal vascular fraction was plated and further cultured as described for BM-MSCs.

After 2 passages, cells were collected and frozen in at least 12 vials. The number of cells in one vial ranged from 3×10^5 to 3×10^6 . In total, 20 different primary cell cultures were

obtained, consisting of 4 groups of 5 samples: (1) 5 BM samples cultured on hPL; (2) the same 5 BM samples cultured on FCS; (3) 5 AT samples cultured on hPL; and (4) the same AT samples cultured on FCS. After freezing of the cells, the vials were distributed on dry ice to 3 laboratories in Leiden, Nijmegen, and Utrecht, respectively.

Reagents

For all culture procedures, the same batch of FCS (HyClone; ARH 27209) and hPL (pool of 35 donors; Sanquin, Utrecht, The Netherlands) was used. All 3 locations used the same brand of culture plastic (Greiner; C7356) and medium (DMEM; Invitrogen) as well as the same kit for isolating RNA (RNeasy Mini kit; Qiagen, Venlo, The Netherlands).

Microarray hybridizations

After thawing one vial, MSCs were seeded in a 180-cm^2 culture flask and expanded until $\sim 80\%$ confluency. In most cases, this confluency was reached within a week. The cell layer was washed with PBS to remove the medium and supplements. Total RNA was isolated from MSCs using the RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. The quality of RNA was tested using the Agilent 2100 Bioanalyzer following the manufacturer's protocol. All samples had a 28S:18S ratio >1.5 , thus passing quality standards for further processing. Two micrograms of total RNA was labeled according to the GeneChip Whole-Transcript (WT) Sense Target Labeling Assay as provided by the manufacturer (Affymetrix, Santa Clara, CA), and hybridized to Human Exon 1.0 ST Arrays overnight before scanning in an Affymetrix GCS 3000 7G scanner. The Human Exon 1.0 ST Array contains more than 1,400,000 probe sets with an average of 4 probes per exon and on average about 40 probes per gene. All hybridizations were carried out in random order at the same facility (Microarray Facility of the Department of Human Genetics, Nijmegen Centre of Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands).

Microarray data handling and statistical analysis

The microarrays were analyzed essentially as previously described [11]. Briefly, Affymetrix CEL files were imported into Affymetrix Expression Console software, to perform quality analysis. Subsequently, CEL files were imported into Partek Genomic Suite Software (Version 6.4; Partek, Inc., St. Louis, MO) and subjected to normalization using the RMA algorithm with GC background correction. To reduce the dataset, signals obtained from the exons of one gene were averaged, reducing the expression data to $\sim 20,000$ genes for one given sample. The 60 samples were analyzed using the same lot of microarrays. The hybridizations of the 60 samples were randomized to exclude day-to-day variations. On the normalized $\log_2$ expression values per gene, a multifactorial analysis of variance (ANOVA) was conducted. Factors CELL CULTURES (cell source), MEDIUM (culture methodology), LOCATION (culture location), and SCAN DATE (hybridization batch) were included in the ANOVA model as independent factors. The obtained P values were corrected for multiple testing by applying the Benjamini–Hochberg method [44]. For each comparison of interest, fold changes

were calculated by dividing the least-square mean expression values of the samples of one group by the least-square mean of the expression values of the comparison group. Fold changes smaller than 1 were replaced by the negative of its inverse.

Relatedness between samples was assessed using principal component analysis (PCA) and Pearson correlation analysis.

Gene ontology analysis of differentially expressed genes

Gene ontology (GO) analyses were performed essentially as described elsewhere [11]. Briefly, lists generated in the pairwise comparisons between the 2 different stem cell sources (AT vs. BM), different laboratories (Utrecht, Nijmegen, and Leiden), and different culture protocols (FCS vs. hPL) were used as input for the online Functional Annotation Tool at the DAVID Bioinformatics Resources (National Institute of Allergy and Infectious Diseases [NIAID], NIH, Bethesda, MD; <http://david.abcc.ncifcrf.gov/>) [45]. Only genes that were significantly ($P < 0.05$) differentially expressed more than 2-fold were included in the analysis. Official HGNP Gene Symbols were used as Gene List input, and all the genes in the human genome served as the background. From the output, only those GO terms that were significantly enriched ($P < 0.05$) were considered. Furthermore, only GO terms that accurately describe biological processes (GO_BP) were further analyzed.

Results

Global analysis of expression data

All 60 samples passed the test for RNA integrity, and all microarrays passed quality control according to Affymetrix Expression Console software. As such, all arrays were included in the analyses, allowing for a high level of robustness in the statistical analysis. Unsupervised hierarchical clustering analysis of array data is depicted in Fig. 1. Based upon this analysis, the most discriminative factor is the source of the MSCs. With a few exceptions, BM-derived cell cultures group together as well as the AT-derived cells. The second parameter that contributes to heterogeneity in the cell cultures is the culture condition. The second column on the left of Fig. 1 shows that within the BM- as well as the AT-derived cells, the samples that are cultured in either FCS or hPL also group together. The third column on the left depicts the effects induced by culturing the same cells at 3 different laboratories. Importantly, laboratory differences do not affect the transcriptomes greatly. The differences are also depicted using PCA. From this analysis, it is clear that BM-derived MSCs cultured in FCS (Fig. 2) show the highest similarity, which is partially lost upon culture in hPL (Fig. 3). For the AT-derived samples cultured in hPL the similarity increases between the individual samples (Figs. 2 and 3). From PCA, it is concluded that BM-MSCs are quite distinct from AT-MSCs.

Comparison of MSCs obtained from BM versus AT

Figure 4 visualizes the correlations between AT-derived MSCs and BM-derived MSCs. The separation of BM-MSCs from AT-MSCs is very distinctive. It is apparent that AT-derived MSCs show a larger heterogeneity, whereas the BM-

derived MSCs are very similar. When BM-derived MSCs are compared with AT-derived cells, 305 genes are 2-fold upregulated in BM-MSCs; 105 genes are 3-fold upregulated; and only 7 genes are more than 10-fold upregulated (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/scd). An unexpected gene, solute carrier family 14 member 1 (*SLC14A1*), a urea transporter, tops the list. It is reported that this gene is expressed in erythrocytes and the kidney. Several of those upregulated genes cannot easily being linked to MSC function. However, others such as vascular cell adhesion molecule 1 (*VCAM1*), fibroblast growth factor receptor 2 (*FGFR2*), and bone morphogenetic protein 4 (*BMP4*) are more likely to have a function in BM-MSCs. Additionally, several members of the integrin family are expressed at higher levels: integrin-alpha 11 (*ITGA11*), integrin-alpha 6 (*ITGA6*), integrin-beta-like 1 (*ITGBL1*), integrin-beta 2 (*ITGB2*), and integrin-beta 8 (*ITGB8*). However, combinations of these alpha- and beta-integrins have not been described yet. A remarkable set of genes that is expressed at a higher level in BM-MSCs concerns some members involved in the WNT-signaling pathway. *WNT5A* and *WNT5B*, but also the receptors *FZD5*, *FZD6*, and *FZD7*, are upregulated, pointing to a prominent role for this pathway in BM-MSCs. Remarkably, also the WNT1-induced protein *WISP1* is expressed at higher levels. *WISP1* is a downstream regulator of the WNT-signaling pathway and attenuates apoptosis [46], while also enhancing osteogenesis [47]. The differences in gene expression between BM- and AT-derived MSCs are not clustered in one part of the genome, but are spread throughout the complete genome (Fig. 5). GO analysis is depicted in Supplementary Fig. S1. Only a few pathways were found to be differentially expressed: (embryonic) skeletal system development, cell adhesion, and neuronal development.

Comparison of MSCs obtained from AT versus BM

The analysis also revealed that, in AT-derived MSCs compared to BM-derived MSCs, 223 genes were expressed more than 2-fold higher, 69 genes expressed more than 3-fold higher, 39 more than 4-fold higher, and 11 more than 10-fold higher in AT-derived MSCs (Supplementary Table S2). For example, early B-cell factor 2 (*EBF2*) is highly upregulated in AT-derived cells, and is a transcription factor that, in osteoblasts, activates the tumor necrosis factor receptor superfamily, member 11b (*TNFRSF11B*), the decoy receptor for receptor activator of nuclear factor kappa-B ligand (*RANKL*), which in turn regulates osteoclast differentiation. This factor synergizes with the WNT-responsive lymphoid enhancer-binding factor 1 (*LEF1*)/catenin (cadherin-associated protein)-beta-1 (*CTNNB1*) pathway. However, in contrast to BM-MSCs, no WNT proteins are upregulated in AT-derived cells. Remarkably, however, the WNT-associated leucine-rich repeat-containing G-protein-coupled receptor 5 (*LGR5*) is expressed at higher levels. *LGR5* is a part of the WNT receptor frizzled and found in many epithelial stem cells. Moreover, the target gene *SFRP1* is also upregulated, indicating a delicate interplay between the Wnt activating (*TNFRSF11B*) and inhibiting mechanisms (*SFRP1*) [48]. Matrix metalloproteinases 1 and 3 (*MMP1* and *MMP3*, respectively) are also higher in AT-derived MSCs. Proteins encoded by the *MMP* genes are involved in the breakdown of the

FIG. 1. Hierarchical clustering of gene expression data. Two-dimensional representation of the unsupervised hierarchical clustering of standardized log₂ gene expression values for 64 cell culture samples. Each row represents a sample and each column a gene. The 785 genes that are represented are differentially regulated (Benjamini Hochberg-corrected $P < 0.05$, fold change > 2) in at least one of the factors, cell cultures, media, or locations. The relative gene expression is visualized by a color gradient as shown by the color bar; *blue* means that the gene is downregulated; *gray* means that there is no change in expression, and *red* indicates upregulation. Color images available online at www.liebertpub.com/scd

FIG. 2. PCA of MSCs cultured in FCS. BM-derived MSCs (*red filled circles*) cluster better than AT-derived MSCs (*blue filled circles*). MSC, marrow stromal cell; BM, bone marrow; FCS, fetal calf serum; PCA, principal component analysis. Color images available online at www.liebertpub.com/scd

FIG. 3. PCA of MSCs cultured in hPL. BM-derived MSCs (*red filled circles*) and AT-derived MSCs (*blue filled circles*). hPL, human platelet lysate. Color images available online at www.liebertpub.com/scd

FIG. 4. Pearson correlation matrix. Depicted are the Pearson correlation coefficients for every combination of samples. The color gradient indicates the relative height of the correlation. Relative low correlations are colored *blue*, whereas very high correlations are shown in *red*. Note that the BM samples correlate higher with each other than with the other samples. RN, AT-derived MSCs; ARH, samples cultured in FCS; Nijm, Nijmegen; Utre, Utrecht; Leid, Leiden. Color images available online at www.liebertpub.com/scd

FIG. 5. Genome-wide locations of the 1134 differentially expressed genes between BM-MSCs and AT-derived MSCs. Chromosome overview showing the location and fold change of the 1134 differentially expressed genes between BM and AT. Each dot represents one gene. The vertical axes represent the base pair position of each gene on the respective chromosome. Fold changes are represented by both the position of the dot on the horizontal axes of the chromosome and a color gradient: a left alignment and blue color indicate downregulated genes; a position close to the middle and gray color are indicative for small changes; and an alignment right from the middle and red color represent upregulation of the gene. RN, AT-derived MSCs. Color images available online at www.liebertpub.com/scd

extracellular matrix in normal physiological processes. As expected, several AT-associated genes, such as the transcriptional regulator of adipocyte differentiation peroxisome proliferator-activated receptor- γ (*PPARG*), were upregulated in AT-MSCs in comparison with BM-MSCs. CD36, upregulated 9.6 times, is a pleiotropic molecule, present on several cell types, and is involved in lipid metabolism, thrombocytopenia, as well as the receptor for *Plasmodium falciparum*. Several genes that have also a role in immune modulation are highly expressed in adipose-derived MSCs: *PTGS1* (*COX-1*) and *HGF* [49]. However, a difference between adipose-derived cells and BM-derived cells is not fully elucidated yet [50]. GO analysis indicates that the pathway affected the most in AT-derived cells is positive regulation of smooth muscle contraction (Supplementary Fig. S2), an unanticipated result.

Comparison of MSCs cultured in FCS versus hPL

The next discriminative factor contributing to MSC heterogeneity is the culture medium used for MSC expansion (Fig. 1). Results were analyzed for BM-derived MSCs (Supplementary Table S3) and AT-derived MSCs (Supplementary Table S4). The largest difference in expression for BM-derived MSCs cultured in FCS was found for aggrecan (*ACAN*), a result not easily explained by differences in the culture medium, but *ACAN* is involved in chondrogenesis. It has been reported that expression of *ACAN* is regulated by *BMP2* in microvascular pericytes [51], suggesting that FCS induces skewing to chondrogenesis. Some unexpected genes

were found upregulated when cultured in FCS: jagged 1 (*JAG1*, also known as *CD339*) and neurogenic locus notch homolog protein 3 (*NOTCH3*), which are both members of one of the main signaling pathways found in stem cells. Culturing of MSCs in FCS induced the expression of noggin (*NOG*) and leukemia inhibitory factor (*LIF*), a cytokine that affects cell growth and development. *NOG* is an inhibitor of *TGF β* signaling, and this inhibition is further enhanced due to the expression of BMP and activin membrane-bound inhibitor homolog (*BAMBI*). *BAMBI* is a pseudoreceptor, lacking an intracellular serine/threonine kinase domain required for signaling. This protein functions as a negative regulator of *TGF β* signaling in frog, mouse, and zebrafish. GO demonstrates that due to culturing in FCS, neurogenesis pathways are negatively regulated (Supplementary Fig. S3).

None of the *WNT* proteins were induced at least more than 2-fold when cultured in FCS compared to hPL. For AT-MSCs cultured in FCS, the same set of proteins was found upregulated as shown for BM-derived MSCs (Supplementary Table S4). Some unique upregulated genes were found. *RELN*, an extracellular matrix serine protease, is known for its role in neurogenesis, but it also binds to the extracellular domains of ApoER2 and VLDLR, both lipoprotein receptors. Also, no *WNT* members are upregulated, but surprisingly a *WNT* receptor-associated protein, *LGR5*, is almost 6 times upregulated.

Comparison of MSCs cultured in hPL versus FCS

The genes that were more than 5 times upregulated in BM-derived MSCs, when cultured in FCS, are not

upregulated when cultured in hPL with one exception. *SERPIN B2* (plasminogen activator inhibitor) is upregulated in FCS, while *SERPIN B7* is upregulated in hPL (Supplementary Table S5). Therefore, a total other set of proteins is induced upon culture in hPL. *Wnt7a* is an illustrative example. The most induced gene is angiopoietin-related protein 4, a protein probably induced by the growth factors present in hPL. It functions as a serum hormone that regulates glucose homeostasis, lipid metabolism, and insulin sensitivity. *PTGS1* or *COX-1* is the enzyme involved in prostaglandin synthesis, which could hint at the immunomodulating effect of MSCs; however, no gross differences exist between FCS-cultured MSCs versus hPL-cultured MSCs in suppressive capacity [32,52].

Adipose-derived MSCs cultured in FCS show a different makeup with regard to the hPL-induced genes (Supplementary Table S6). Two ABC transporters, ATP-binding cassette subfamily A (*ABC1*), member 9 (*ABCA9*), and ATP-binding cassette subfamily A (*ABC1*), member 6 (*ABCA6*), are expressed at higher levels in adipose-derived MSCs when cultured in hPL. *CD36* is clearly induced in adipose-derived MSCs when cultured in hPL. *COLEC12*-collectin subfamily member 12, a C-type lectin, is upregulated in hPL-cultured cells irrespective their origin (BM or AT). GO analysis revealed that several pathways involved in immune regulation are induced by hPL (Supplementary Fig. S4).

The expression pattern of BM- versus AT-derived MSCs when cultured in FCS

The differences in the cell source when cultured under identical conditions are shown in Supplementary Tables S7 and S8. The difference in gene expression is due to the different tissue microenvironment where the MSCs are collected from. The *TSPAN 18* that tops the list is an understudied tetraspanin most probably involved in Ca signaling. The large number of genes indicates that large differences exist between the 2 MSC populations. The genes overexpressed in AT-derived MSCs compared to BM-derived MSCs are much smaller. Some remarkable genes were found. Fourteen genes are over 10 times more expressed in AT-derived MSCs as compared by their BM counterparts (Supplementary Table S8).

The expression pattern of BM- versus AT-derived MSCs when cultured in hPL

The most prominent difference between BM- and AT-derived MSCs is Hyaluronan and proteoglycan link protein 1, a protein expressed more than 60 times higher in BM-MSCs when cultured in hPL. Remarkably, also *BMP4* is highly expressed in BM-MSCs (Supplementary Table S9). Some genes are expressed independently from the culture method such as *TSPAN18* and *VCAM-1*. The same holds for AT-derived MSCs cultured in hPL: *CD36*, *PEG10*, and *ADAMTS15*. *LGR5* is a typical example of a gene expressed in AT-MSCs, but at a higher level when cultured in FCS compared to hPL (Supplementary Table S10).

Differences induced by culturing in different laboratories

Cells were thawed in 3 laboratories at different locations in The Netherlands: Leiden, Utrecht, and Nijmegen. MSCs

were seeded and expanded until 80% confluency was reached. Microarray analysis indicated that the cultures were very similar. Especially, between Leiden and Nijmegen, only a few genes showed differential expression (Supplementary Table S11). Pyruvate dehydrogenase kinase isozyme 4 (*PDK4*) was induced more than 2-fold in Nijmegen, as compared to Leiden and Utrecht. FBJ murine osteosarcoma viral oncogene homolog (*FOS*) is downregulated in Nijmegen compared to Leiden and Utrecht. Obviously, the number of differentially expressed genes is too small to perform a reliable GO analysis.

The differences between Utrecht and the other 2 locations were larger. Laminin 4 (*LAMA4*) is expressed at lower levels in Utrecht compared to Nijmegen and Leiden. Several genes are upregulated compared to Leiden and Nijmegen. These genes are dual-specificity phosphatase 5 and 6 (*DUSP5* and *DUSP6*, respectively), adhesion molecule with Ig-like domain 2 (*AMIGO2*), hairy and enhancer of split 1 (*HES1*), sprouty homolog 2 (*SPRY2*), Krüppel-like factor 4 (*KLF4*), growth arrest and DNA-damage-inducible-45-beta (*GADD45B*), and SERTA domain-containing 1 (*SERTAD1*). The latter 2 are both involved in cell growth. For the difference between Utrecht and Nijmegen and Utrecht and Leiden, a GO analysis was performed. Between Utrecht and Leiden, negative regulation of kinase activity constituted the main difference (Supplementary Fig. S5), while the main difference between Utrecht and Nijmegen was response to protein stimulus (Supplementary Fig. S6). The difference between Leiden and Utrecht was confined to one fatty acid metabolic process (Supplementary Fig. S7). The difference between Nijmegen and Utrecht was mainly found in immune response pathways (Supplementary Fig. S8). Donor origin of the cell cultures proved to be the least discriminating factor. Although some marked differences are visible in the PCA, no discrete clusters were observed (Supplementary Fig. S9).

Discussion

Heterogeneity of MSCs is a well-known phenomenon that has been put forward to explain success or failure of some MSC preparations in tissue regeneration. Heterogeneity has been linked to the outgrowth of certain subsets driven by minute differences in culture conditions. Although different subsets with specific characteristics were isolated, those subsets were present in most MSC samples. Besides the outgrowth of certain subsets, transcriptomes are also changed due to culture of the cells using those different conditions [53–56]. Presently, it is unknown whether in some donors some of the subsets fail to grow in vitro, which could explain the lack of tissue regenerating capacity in some cases. Here, we show that the most discriminating factor is the source of the MSCs. The fact that in vitro differentiation assays show similar results. Previously, we demonstrated that AT-derived MSCs display a different transcriptome compared to BM-MSCs [11]. Now, we expand this finding by showing that the tissue source has a greater influence on gene expression profiles than the different culture methods (FCS vs. hPL). It seems feasible that heterogeneity arises from the way of collecting BM: it is an invasive procedure that destroys the local environment and disrupts many blood vessels. Disruption of BM could lead to recovery of different subsets (BM-harvesting site and BM aspirate volume). For

example, when more blood vessels are disrupted, the chances that pericytes are collected that are present in the vessel walls are higher. Since pericytes are considered early precursors of MSCs, this could induce a donor-to-donor variability in heterogeneity [57,58]. Although AT collection is also a harsh procedure, the AT, at first sight, seems a more homogeneous tissue than BM. It is therefore remarkable that the highest level of heterogeneity was detected in the AT-derived MSCs. We collected AT from patients who underwent joint surgery. One has to keep in mind that AT obtained from other locations could have a slightly different transcriptome [59,60]. The higher level of heterogeneity of AT-derived cells suggests that those cells do represent MSCs that have more lineage capabilities than BM-MSCs. Indeed, BM-MSCs are more committed to the bone and cartilage lineage than AT-derived cells [61–63]. Others showed chondrogenesis with adipose-derived cells [50]. However, the lineage capabilities are dependent on the growth factor added to the culture and the space available for the cells [64]. The difference in chondrogenesis between BM and AT is fully lost by adding BMP6 or GDF-5 [65,66].

The next level of heterogeneity was imposed by using either FCS or hPL. Apparently, FCS has a negative regulatory effect on neurogenesis. Notwithstanding this feature, MSCs can stimulate neurogenesis, not by differentiating into neuronal tissue, but by supplying neurological growth factors [67–69]. Based on our results, a better option for procedures aimed to improve neurogenesis is to use MSCs cultured in hPL. Several genes involved in immunity are expressed at higher levels when MSCs are cultured in hPL. This higher expression of immunity-related genes nicely fits in the observation that platelet-expanded MSCs have less immunosuppressive capacity than MSCs expanded in FCS [69,70].

Remarkably, the culture location did not induce a large degree of heterogeneity. Two locations hardly showed significant differences, but comparison with the third location yielded some differentially expressed genes. In one location, immune-response genes were upregulated, but this was not the case when compared with the other location. However, no other clearly affected pathways could be assigned to these differences. Apparently, MSCs have a fairly robust transcriptome when the same culture conditions are applied in different laboratories. Another factor driving heterogeneity is donor-to-donor differences. It was reported earlier that donor-to-donor differences are minor [11], and this was confirmed in the present study with a large set of unrelated donors. The highest level of similarity was detected in the BM-derived samples. Finally, sex-derived differences [71,72] could be excluded, since in both donors sets, the male–female ratio was comparable.

In conclusion, this robust study shows that the most discriminative feature for heterogeneity within MSC cultures is the tissue source, followed by the culture methodology. Although differences were found between the laboratories, cell culture on 3 different locations caused only a low degree of heterogeneity. Finally, individual donors contributed marginally to the observed differences in transcriptomes. BM-derived MSCs displayed the highest level of similarity irrespective their culture history when compared to AT-derived MSCs.

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Author Disclosure Statement

The authors declare no competing financial interests in conjunction with the study described in this manuscript.

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