Commentary

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Epigenomics

Epigenetic quality check – how good are your mesenchymal stromal cells?

"Particularly with regard to clinical trials – but also for basic research – reliable quality control of cell preparations is a prerequisite for reproducible results."

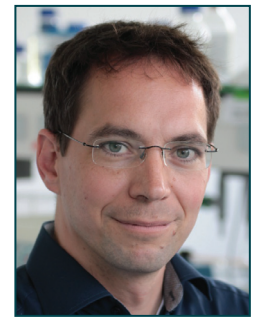
First draft submitted: 5 May 2016; **Accepted for publication:** 12 May 2016; **Published online:** 1 July 2016

Keywords: cellular therapy • DNA methylation • epigenetics • heterogeneity • mesenchymal stem cells • mesenchymal stromal cells • quality control • senescence

Mesenchymal stromal cells (MSCs) raise high hopes for tissue engineering and cellular therapy. They are concurrently tested in more than 600 clinical trials for a wide variety of diseases [1]; however, most of these studies are still in Phase I or II and only a few have so far clearly proven that MSCs provide a therapeutic benefit [2]. Cellular therapeutics are pharmaceutical products which need to be generated under standard operating procedures and according to strict guidelines of GMP. In this light, it is surprising that a reliable molecular definition and quality control of MSCs are yet elusive. MSCs can be isolated from various tissues, with different isolation and culture expansion methods – and this provides many variables to the already existing high interindividuality within MSC preparations. Furthermore, the cells notoriously acquire continuous changes during culture expansion. So far, biomarkers remain elusive to clearly discern the subset of multipotent cells which is capable of multilineage differentiation toward various mesodermal cell types. Particularly with regard to clinical trials – but also for basic research – reliable quality control of cell preparations is a prerequisite for reproducible results. In this commentary, we will demonstrate that epigenetic analyses provide a new perspective to ultimately reach this goal.

Current state of quality control of MSCs

Since the first description of MSCs by Friedenstein in the 1960s [3] great efforts have been made to identify specific parameters for characterization of MSCs. The International Society for Cellular Therapy proposed minimal criteria for the definition of MSCs in 2006 which are still considered state of the art [4]: MSCs grow plastic adherent with fibroblastoid morphology; they possess *in vitro* differentiation capacity into at least osteogenic, adipogenic and chondrogenic lineages – which is, however, difficult to quantify and may not reflect *in vivo* differentiation potential; and they express the surface markers CD105, CD73 and CD90, whereas hematopoietic markers, such as CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR, are not expressed. In addition, colony forming unit frequency, proliferation potential, viability, karyotypic analysis and absence of viral or microbial contaminants are often controlled. These criteria facilitate a certain level of standardization – but they neither reflect the functional differences that are known to exist between different MSC preparations, nor do they provide insight into the heterogeneity within MSC preparations. All the more it is important to provide the minimum information about MSC preparations in scientific studies – including species, donor type (autologous or allogeneic), tissue type of origin, donor age, harvesting procedure, isolation methods, culture conditions and the number of passages (or even cumulative population doublings). However, this information is not provided in many manuscripts making it difficult to recapitulate results.



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To better standardize MSC preparations, it has been proposed to utilize a well-defined reference cell material that can be used to track consistency and potency of MSCs [5]. This reference cell material needs to be well characterized, widely available at low costs and resistant to changes evoked by replicative senescence. Large pools of MSCs from different donors, immortalized cell lines or induced pluripotent stem cell (iPSC)-derived MSCs [6] might be suitable as reference cell material. Further development of an appropriate reference would require close collaboration of scientists, governmental agencies, foundations and industry. More recently, Hematti proposed a combination of biological and analytical assays, including mixed-lymphocyte reactions, gene expression profiles or protein-based analysis of the MSC secretome, to assess the potency of MSCs [7]. It remains to be demonstrated if reference cell preparations or combinations of functional assays are ultimately applicable in daily routine for quality control of MSCs.

The cell-type is defined by the epigenetic makeup & *vice versa*

Cellular differentiation is governed by epigenetic modifications, which impact on chromatin structure and regulate accessibility of specific genomic regions. During developmental processes and cellular differentiation epigenetic modifications occur in a highly reproducible manner – and these epigenetic changes ultimately determine the difference between cell types. In principle, epigenetic patterns are reversible, but they appear to be relatively stable in comparison to gene expression patterns which are often more directly influenced by culture conditions. The different types of epigenetic modifications probably act in concert. DNA methylation (DNAm) at specific cytosine guanine dinucleotides (CpG sites) depicts the so far best understood epigenetic modification. In contrast to other epigenetic alterations, such as covalent modification of histone tails, DNAm levels can be reliably quantified on single nucleotide resolution. Methods for genome-wide DNA methylation analysis include whole-genome bisulfite sequencing, reduced representation bisulfite sequencing and microarrays such as the Illumina HumanMethylation BeadChips. Furthermore, locus-specific analysis of a small number of CpG sites is possible by more time-saving and cost-effective approaches, such as pyrosequencing or MassARRAY analysis of PCR amplicons of bisulfite converted DNA. DNAm patterns are modified by DNA methyltransferases in the course of differentiation and in disease – but our understanding of how these modifications are regulated site specifically in the genome is sparse. Furthermore, it is still not understood whether or not DNAm is functionally relevant, *per se* [8]. Either way, the highly reproducible cell type-specific epigenetic differences – which are apparently directly involved in cellular specification – and the availability of methods for precise site-specific quantification make DNAm patterns ideal candidates for molecular characterization and definition of cell types.

How to discern fibroblasts & MSCs?

MSCs were originally isolated from the bone marrow as fibroblast-like colony forming cells. Despite intensive research over half a century the molecular classification into MSCs and fibroblasts still remains a challenge [9]. Both cell types can be isolated from various tissues, and they reveal very similar morphology, proliferation, growth pattern and immunophenotype. Most authors indicate that particularly MSCs comprise a multipotent subset capable of *in vitro* and *in vivo* differentiation toward at least osteogenic, adipogenic and chondrogenic lineages – which is why these cells are often alternatively termed as 'mesenchymal stem cells'. For definition of fibroblasts this multilineage differentiation potential is not a prerequisite, but it has to be taken into account that the *in vitro* differentiation assays are artificial, they hardly reflect *in vivo* differentiation potential and they are difficult to standardize and quantify. Therefore, it is not trivial to discern fibroblasts and MSCs – and to proof that the two types of cell preparations are actually not the same.

> "Alternatively, mesenchymal stromal cells and fibroblasts can be classified by epigenetic means."

Gene expression profiles, such as PhysioSpace, can be used to provide cell type-specific signatures that are based on large sets of publicly available gene expression data [10]. This method allows reliable classification of distinct cell types even in case of small sample sizes. A newly established and more MSC-specific classification approach is the Rohart MSC Test that is based on a 16-gene signature allowing classification of MSCs with >97% accuracy and demarcation from fibroblasts and other somatic cells [11]. However, these approaches for cellular characterization necessitate analysis of gene expression profiles, normalization procedures and rather complicated bioinformatics. Furthermore, RNA is relatively prone to degradation and gene expression is highly influenced by culture conditions and cell cycle progression.

Alternatively, MSCs and fibroblasts can be classified by epigenetic means. Our laboratory has recently described an Epi-MSC-Score [12]: to identify relevant genomic regions that discern MSCs and fibroblasts, we utilized 83 DNAm profiles from 12 studies for a

training data set and 107 DNAm profiles of 16 studies as validation data set. Thereby, two CpG dinucleotides were identified – associated with *C3orf35* and *CIDEC* – that are higher methylated in MSCs or fibroblasts, respectively. The Epi-MSC-Score is determined as difference of these DNAm levels: a level above 0 is indicative for MSCs. Pyrosequencing assays were established for the two relevant CpGs to facilitate fast and cost-effective analysis of DNAm levels [12]. These assays are fully described and can easily be recapitulated by other scientists – alternatively, service for this analysis can be provided by Cygenia [13]. The Epi-MSC-Score supports classification of cell preparations into MSCs and fibroblasts – according to the classification of 28 previous studies by other groups – but it is yet unknown if this measure correlates with the clinical potential of cell preparations.

MSCs from different tissues are not the same

Although MSCs were initially isolated from bone marrow, very similar cell preparations have since then been described in a multitude of different tissues using the same acronym – including adipose tissue, cord blood, umbilical cord and placenta. MSCs have been suggested to resemble perivascular cells and therefore similar cells may reside in any vascularized tissue [14]. In fact, MSCs from different tissues are virtually undistinguishable in morphology and proliferation and they usually display an overall quite similar immunophenotype. However, there is a growing perception that MSCs isolated from different anatomical locations reflect unique biological properties *in vitro* [15] and *in vivo* [16]. For example, MSCs from bone marrow have higher osteogenic and chondrogenic differentiation potential [16], hematopoiesis supportive function [17] and different cytokine secretion [17] as compared with MSCs from adipose tissue.

Immunophenotypic marker panels have been suggested to define the tissue of origin of MSCs [18]; however, a common and applicable set of surface markers is still elusive and expression of surface markers changes during culture expansion [19]. Gene expression profiles reveal significant differences between MSCs of different tissues [15]. Furthermore, DNAm profiles provide very significant differences between MSCs from different tissues, too [16,20]. For dermal fibroblasts, we have even demonstrated that the anatomical location (e.g., arm, leg and abdomen, among others) is reflected in global DNAm profiles after three passages [21] – conversely, it can be speculated that there are also significant differences in MSCs isolated from different adipose tissues throughout the body. To better classify MSC preparations according to their tissue type of origin, our group described an Epi-Tissue-Score – in analogy to the Epi-MSC-Score – which facilitates fast and robust classification of MSCs that are either derived from bone marrow or adipose tissue: DNAm levels are analyzed at two CpG sites that are associated with *SLC41A2* and *TM4SF1* – which are methylated in MSCs from bone marrow or adipose tissue, respectively. The Epi-Tissue-Score is determined as the difference of these two DNAm levels (a level above 0 is indicative for MSCs from bone marrow) [12]. As mentioned above, MSCs from adipose tissue and bone marrow differ significantly in their function and therefore this type of quality control may be useful in some experimental settings. Furthermore, it may also be interesting to analyze whether or not tissue-specific patterns change upon transplantation of MSCs to other sides in the organism.

Impact of culture conditions on the epigenetic makeup

Primary MSCs can be directly isolated from tissues without any *in vitro* culture by selection via specific surface markers, such as CD271 [18] – but these approaches facilitate only a crude enrichment, whereas generation of relevant cell numbers consisting of pure MSCs usually necessitates culture expansion over several passages. On the other hand, different culture conditions – such as oxygen pressure [22], seeding density [23] and culture media – may evoke molecular sequels and therefore need to be considered. So far, fully defined synthetic culture conditions remain elusive and culture media are usually supplemented with either fetal bovine serum or human platelet lysate [24]. It has been suggested that these serum supplements may result in altered expression of surface markers and inhibitory activity on T-cell and NK-cell proliferation [25]. Furthermore, a serum free culture medium resulted in decreased CD105 expression [26]. To date, very little is known about functional and epigenetic consequences of culture conditions. It is, however, expected that at least the heterogeneous composition of subpopulations is influenced by culture procedures – and if so, then this should be monitored by specific biomarkers for additional quality control.

Senescence-associated DNA methylation changes

It is virtually impossible to fully standardize cell preparations – especially, because primary cells undergo continuous changes from the moment of culture-isolation. Replicative senescence was first described by Leonard Hayflick in the 1960s [27] and it is still unclear how this process is mediated and if it is related to aging of the whole organism [28]. Either way, long-term culture has

major functional implications on proliferation, morphology, secretory profile, immunomodulatory properties and differentiation potential of MSCs [29]. Furthermore, senescent cells reveal telomere attrition and possibly accumulation of DNA damage. Therefore, it is highly relevant to take the state of replicative senescence into account, when evaluating MSC preparations for clinical applications – but also for basic research.

> "We have described an Epigenetic-Senescence-Signature that is based on six CpG sites associated with the genes *GRM7*, *CASR*, *PRAMEF2*, *SELP*, *CASP14* and *KRTAP13–3*."

Senescent cells encompass a phenotype characterized by specific features in gene expression, senescence-associated beta galactosidase activity, senescence-associated heterochromatic foci, DNA damage foci and a senescence-associated secretory phenotype. Notably, cellular aging during *in vitro* culture is also associated with highly reproducible DNAm changes, particularly in developmental genes [20,30]. Some CpG sites reveal almost linear changes in DNAm over subsequent passages and can therefore be utilized to track culture expansion. We have described an Epigenetic-Senescence-Signature that is based on six CpG sites associated with the genes *GRM7*, *CASR*, *PRAMEF2*, *SELP*, *CASP14* and *KRTAP13-3* [31]. To date, this approach seems to provide the most accurate possibility to estimate cumulative population doublings and passage numbers. The method has already been tested for therapeutic cell preparations, which were generated under GMP conditions [32].

In analogy, aging of the organism is also reflected by very specific DNAm changes. Various epigenetic models have been proposed to estimate donor age in different tissues [33,34]. The precision of these models is impaired by molecular changes during culture expansion – but it may still provide an estimate for the chronological age of the donor [6]. It has to be pointed out that senescence-associated DNAm changes differ from age-associated DNAm changes – and therefore both processes can be tracked by corresponding epigenetic signatures. Notably, age-associated as well as senescence-associated modifications are entirely reversed by reprogramming into iPSCs [35]. Upon redifferentiation of iPSCs toward MSCs the age-associated epigenetic changes are not reacquired, whereas senescence-associated DNAm accumulates again from the time point when the cells exit the pluripotent state [6].

Future perspective

The presented epigenetic signatures are ideally suited to better characterize MSCs – DNA methylation is directly linked to differentiation toward specific cell types, it is relatively stable, DNA can be easily harvested and shipped at room temperature and DNAm levels can be quantified precisely on single base resolution. Further development of tailored epigenetic signatures will be important to better understand the impact of culture conditions – which has so far hardly been addressed.

There is a growing perception that MSCs comprise subsets of different proliferative potential, morphology, immunophenotype and gene expression [36]. Intercellular heterogeneity is also reflected in the senescent state of MSC subpopulations during culture expansion [37]. It will be of particular relevance to identify epigenetic characteristics of the real 'mesenchymal stem cell' fraction with multipotent differentiation potential. To this end, simultaneous analysis of functional parameters and epigenetic profiles will be required, but such analysis is still hampered by the relatively high number of required cells. On the other hand, advances in microfluidics may even facilitate single-cell analysis. Single-cell RNA-seq in MSCs demonstrated differential expression of lineage-specific genes [38]. Next-generation sequencing approaches may also facilitate reliable analysis of DNAm in individual cells [39]. Such technical advances enable epigenetic profiling of MSC subpopulations in comparison to the bulk population to better understand the molecular heterogeneity and to ultimately determine the stem cell fraction.

More important than molecular standardization of MSCs are biomarkers that are indicative for their therapeutic potential. Such biomarkers could be used to further optimize culture conditions – and to ultimately provide a quality control that is directly related to therapeutic potency. However, such epigenetic signatures can only be identified retrospectively by analysis of DNAm profiles in samples from clinical trials. It is therefore important to compare MSC preparations with and without therapeutic response to identify suitable biomarkers. This knowledge may also provide insight into the molecular and cellular mechanisms of cellular therapy – and thereby help to increase the success rates of clinical studies with MSCs in the future.

Financial & competing interests disclosure

W Wagner is cofounder of Cygenia GmbH and R Goetzke contributes to this company, too. Cygenia provides service for the Epi-MSC-Score, the Epi-Tissue-Score and the Epigenetic-Senescence-Signature to other interested scientists (www.cygenia.com). This work was supported by the Else Kröner-Fresenius Stiftung (2014_A193), by the German Ministry of Education and Research (BMBF; OBELICS) and the

START-Program of the Faculty of Medicine, RWTH Aachen. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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