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Title

Transcriptomics of Human Multipotent Mesenchymal Stromal Cells: Retrospective Analysis
and Future Prospects

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Abstract

The plastic-adherent, fibroblast-like, clonogenic cells found in the human body now defined as multipotent “Mesenchymal Stromal Cells” (MSCs) hold immense potential for cell-based therapies. Recently, research and basic knowledge of these cells has fast-tracked, both from fundamental and translational perspectives. There have been important discoveries with respect to the available variety of tissue sources, the development of protocols for their easy isolation and *in vitro* expansion and for directed differentiation into various cell types. In addition, there has been discovery of novel abilities such as immune-modulation and the further development of the use of biomaterials to aid isolation, expansion and differentiation together with improved delivery to the selected optimal tissue site. However, the molecular fingerprint of MSCs in these contexts remains imprecise and inadequate. Consequently, without this crucial knowledge it is difficult to achieve progress to determine with precision their practical developmental potentials. Detailed investigations on the global gene expression, or transcriptome, of MSCs could offer essential clues in this regard. In this article, we address the challenges associated with MSC transcriptome studies, the paradoxes observed in published experimental results and the need for careful transcriptomic analysis. We describe the exemplary applications with various transcriptome platforms that are used to address the variation in biomarkers and the identification of differentiation processes. The evolution and the potentials for adapting next-generation sequencing (NGS) technology in transcriptome analysis are discussed. Lastly, based on review of the existing understanding and published studies, we propose how NGS may be applied to promote further understanding of the biology of MSCs and their use in allied fields such as regenerative medicine.

Keywords: Mesenchymal Stem Cells; Phenotype; Gene Expression; Stemness; Differentiation; Transcriptomics; Micro Array; Next-Generation Sequencing; Biomaterials; Regenerative Medicine

Abbreviations: MSCs, multipotent mesenchymal stromal cells; AD-MSCs, adipose-derived MSCs; BM-MSCs, bone marrow-derived MSCs; PL-MSCs, placenta-derived MSCs; FL-MSCs, fetal-derived MSCs; NSCs, neural stem cells; HSCs, hematopoietic stem cells; ESCs, embryonic stem cells; NGS, next-generation sequencing; RNA-seq, RNA sequencing; mRNA, messenger ribonucleic acid; cDNA, complementary deoxyribonucleic acid; DEG, differentially expressed genes; FPKM, fragments per kilo base million; RPKM, reads per kilo base million; ISCT, international society for cellular therapy; TGF, transforming growth factor.

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1. Introduction

Seminal studies involving bone marrow transplants in a patient suffering from the blood cancer condition leukaemia were first performed by Edward D. Thomas during the 1950s (Little and Storb, 2002). These investigations together with those by Robert A. Good in the 1960s for treatment of the non-cancer condition severe combined immunodeficiency syndrome prompted the belief that bone marrow possesses a unique pool of cells with the potential ability to cure human blood diseases (Gatti et al., 1968; Thomas et al., 1957). Subsequently, classical studies by Alexander J. Friedenstein during the 1970s and 80s, identified the non-haemopoietic cells in the marrow as adherent, fibroblast-like, clonogenic cells. These cells showed high “replicative capacity” *in vitro* and had the “ability to differentiate” into osteoblasts, chondrocytes, adipocytes and hematopoietic-supporting stroma *in vivo* (Friedenstein et al., 1970; Friedenstein et al., 1966). This unique pool of cells, were initially termed “colony forming unit-fibroblastic” but are now popularly defined as “Mesenchymal Stromal Cell” (MSCs). They were first isolated from bone marrow as 0.001-0.01% of the total nucleated cells and it was proposed that these cells were part of a wider stromal cell system of the body and that stromal fibroblastic cells were present in many other tissues (Owen, 1988). However, from much definitive work it was shown that only the bone marrow contained MSCs that had the inherent capacity to differentiate into all lineage-specific component cells of the osteoblastic lineage and were termed by Friedenstein as “determined osteoprogenitor cells”. Other stromal fibroblastic cells, either from the bone marrow or from a large spectrum of other tissues, could only be induced into the osteogenic lineage by specific inductive molecules and these were termed “inducible osteogenic progenitor cells” (Friedenstein, 1973). Since then, other non-marrow tissues harbouring MSCs that are more easily obtainable and accessible, such as adipose and umbilical cord tissues, have been studied in more detail (Bennett et al., 1991; Fotia et al., 2015; Hass et al.,

2011; Minguell et al., 2001; Pierantozzi et al., 2010; Riekstina et al., 2009; Sharma and Bhonde, 2015; Strioga, Marius et al., 2012). Simultaneously, novel protocols have continuously been developed to differentiate these MSCs *in vitro* into lineage-specific cell types (Beresford et al., 1994; Gundle et al., 1995; Marie and Fromigué, 2006; Oreffo et al., 1997; Xiong et al., 2014).

The notion that MSCs of a tissue origin can differentiate into a particular set of progeny only was taken over by fascinating and controversial investigations that introduced a phenomenon termed “trans-differentiation” to turn “brain into blood” (Bjornson et al., 1999), “blood into brain” (Mezey et al., 2000) and other cross-lineage differentiation (Krause et al., 2001). In addition, several other noteworthy investigations revealed the immune-suppressive/modulatory/regulatory abilities of MSCs (Caplan, 2007; Fiorina et al., 2009; Ren et al., 2008; Siegel et al., 2009). This led to an increased excitement about the possible potential of MSCs in treatments for various other diseases or conditions. Taken together, it was evident that a) the discovery of various tissue sources of adult stem cells, b) the development of defined protocols for their easy isolation, culture and differentiation into lineage-specific cell types, c) the design of novel strategies for induction of differentiation of these cells into a variety of cell types other than found in their tissue of origin, and d) discovery of their novel abilities, such as immune-modulatory ability, has elevated MSCs to the forefront of stem cell research activities. The fascination of these cells relates both to their fundamental nature as well as to their translational possibilities (**Figure 1**). However, even now MSC research is still at an early phase with high potential but with many remaining questions. Major challenges include: isolation with high accuracy and viable yield, *in vitro* expansion without losing stemness characteristics and efficient differentiation protocols in 2D cultures (i.e. conventional two-dimensional cell cultures systems) and in 3D cultures (i.e. three-

dimensional cell culture systems developed using biomaterials that resemble physiological environments).

Detailed investigations on the global gene expression or transcriptome of MSCs could possibly offer essential clues to further our understanding on MSCs. A single transcriptome is a snapshot of all the active genes at a given time. When a series of transcriptomes is obtained, comparative transcriptome analysis can be performed to detect trends of gene expression changes in response to a treatment or a native variable. Gene expressions in an individual sample need to be normalized, for example to housekeeping genes, or by a mathematical formula, to enable quantitative or semi-quantitative comparison among samples for identification of DEGs due to a treatment or a biological time course. A typical transcriptome approach requires the total mRNA population of a sample as the starting material. From total RNA extracts, the commonly used method is to apply oligo-dT probes for mRNA isolation followed by biochemical conversion to cDNA which will be examined by gene identification technologies such as microarray (**Figure 2**).

Early comparative transcriptome work with MSCs demonstrated molecular similarities between human AD-MSCs and BM-MSCs (Katz et al., 2005), as well as population plasticity and biochemical heterogeneity (Phinney, 2007, 2009; Phinney and Isakova, 2005). By comparing transcription patterns of MSCs and MSC-like cells, isolated and cultured under identical conditions, the expression pattern was identified to be cell-type-specific rather than governed by isolation and culture conditions (Ulloa-Montoya et al., 2007). However, culture media with or without fetal bovine serum as a nutritional supplement affected the MSC transcriptome stability during *in vitro* expansion (Shahdadfar et al., 2005). Human BM-MSCs cultured in two different expansion media (BM-MSC-M1 and BM-MSC-M2) displayed changes in their gene expression profiles (Wagner, W. et al., 2006). In addition, culture duration has been found to affect MSC biology; long-term *in vitro*

expansion has altered gene expressions of adult MSCs (Izadpanah et al., 2008). Comparative transcriptome analysis also determined MSC-disease associations. For example, differential expression of genes reflected impaired clonogenic and proliferative potentials determined in BM-MSCs isolated from patients with active rheumatoid arthritis when compared with those from healthy donors (Kastrinaki et al., 2008).

To this end, we believe that the detailed investigation of the transcriptome of MSCs could potentially offer invaluable solutions. High-throughput technologies such as microarray analysis do trigger much interest for transcriptomics studies in general, but the associated high costs and technical limitations remain restricting factors. With the advent of relatively cost-effective and robust technologies, such as NGS, the field of transcriptomics is gaining momentum. We believe, therefore, that it is time to make use of such emerging technologies to overcome the bottlenecks in MSCs research. In this article, we discuss the necessity for global gene expression analysis of MSCs. This is followed by a description of the various methods and tools available for transcriptome analysis. We continue by a description of a few examples of investigations that have been carried out on this topic so far and conclude by discussing the future prospects for transcriptome analysis of the MSC system.

2. Need for a global view of MSCs gene expression

2.1. Deciphering the identity code

With the overwhelming expansion of research on MSCs, there was ambiguity not only in the nomenclature but also in the identification of these cells. After defining the fibroblast-like plastic-adherent cells, regardless of the tissue of origin, as “multipotent mesenchymal stromal cells” in the year 2005, the Mesenchymal and Tissue Stem Cell

Committee of ISCT proposed three minimal criteria for defining the multipotent MSCs in year 2006 (Dominici et al., 2006). *“First, MSC must be plastic-adherent when maintained in standard culture conditions. Second, MSC must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR surface molecules. Third, MSC must differentiate to osteoblasts, adipocytes and chondroblasts in vitro”* (Dominici et al., 2006). Yet, numerous investigations on MSCs have reported several deviations from the ISCT minimal criteria, mainly due to isolation, anatomical origin and donor-related issues. For example, MSCs have been traditionally isolated utilizing their plastic adherence property and identified using a set of positive and negative surface markers as described by ISCT; this, however, would retain various other types of cells besides MSCs (Baustian et al., 2015; Ramakrishnan et al., 2013). Even when the contaminating cell population was eliminated by using improved isolation and enrichment procedures, heterogeneity was reported within the isolated MSC population (Wagner, Wolfgang et al., 2006). Moreover, differences in the isolation procedures were found to yield MSCs with different phenotypes (Seeger et al., 2007). With respect to the anatomical origin, significant differences have been observed in the molecular phenotype between MSCs derived from bone marrow, adipose tissue and skin, suggesting ontological and functional differences (Al-Nbaheen et al., 2013; Strioga, Marius et al., 2012). In addition, with differences in donor-age and gender, three surface markers (CD119+, CD146+, HLA ABC+) have been identified in BM-MSCs that are partially donor-related (Siegel et al., 2013). Taken together, these examples suggest that isolation procedures, tissue origins and donor-related differences all contribute to the challenges in precise MSC identification. This uncertainty imposes difficulties when comparing results from different studies but also restricts the use of MSCs in clinical applications. Thus, a universally agreed definition for MSCs, in terms of identification, is still needed.

2.2. Addressing the paradoxes of expansion

In a typical bone marrow graft, only 0.0002 – 0.0005% of extracted mononuclear cells are estimated to be MSCs, which amounts to 400 – 1000 MSCs/kg. The isolated MSCs need to be expanded 10 – 13.6 fold to satisfy the therapeutic requirements of $1 - 5 \times 10^6$ MSCs/kg-patient-weight (Koc and Lazarus, 2001). Fortunately, it is relatively easy to culture MSCs *in vitro* and extensive research on their expansion has led to the development of novel procedures for the production of MSCs in terms of quality (i.e. homogeneity of population) and quantity (i.e. yield per extraction) (Lim et al., 2007). For example, it has been reported that MSCs were able to be expanded up to 22 passages without losing stemness (Estrada et al., 2012; Pierantozzi et al., 2010). Expansion of MSCs under atmospheric CO₂ conditions has been noted to avoid the effects of the bicarbonate buffering system (Brodsky et al., 2013). The long-term culture of MSCs under 5% O₂ conditions, which represents hypoxia *in vitro* but normoxia *in vivo*, has been observed to avoid the aging issues associated with standard 21% O₂, which is normoxia *in vitro* but hyperoxia *in vivo* (Basciano et al., 2011). However, it has been discovered that the BM-MSCs exhibit impaired functionality and lose differentiation potential from passage 6 and are prone to telomere shortening from 9.19 Kbp to 8.7 Kbp. Therefore, these cells show the typical Hayflick phenomenon of cellular aging after passage 9 (Bonab et al., 2006). Moreover, *in vitro* expansion was also associated with the onset of heterogeneity in the resultant MSCs as reported by (Whitfield et al., 2013). Such an expansion-associated or extrinsic heterogeneity greatly adds to “MSCs genuineness” issues that are already clearly apparent due to the isolation-associated or intrinsic heterogeneity of these cells. Given the evidence that MSCs begin to lose their specific stem cell features virtually from the moment *in vitro* culture begins, it is important for validation of any expansion methodologies that have been reported or may emerge in the future to characterize the cultured MSCs both at the cellular level and also the molecular level.

2.3. Understanding emerging trends in stem cell research

With the advent of biomaterials, an enormous number of investigations are now being reported on the use of biomaterials in stem cells research in a variety of contexts (Barralet et al., 2005; Xia et al., 2005; Xu et al., 2012; Xu et al., 2014; Zhu et al., 2010). For instance, the use of silicone tubes conjugated with anti-CD34 antibody has been described for the isolation of MSCs from traditional crude preparations as a means to reduce the heterogeneous cell pool (Mahara and Yamaoka, 2010). Other reports, have demonstrated the use of poly(lactide-co-glycolic acid) based micro-particles conjugated with CD34 for the isolation of endothelial progenitor cells from human peripheral blood, as a means to overcome the scale-up issues associated with conventional fluorescence-activated cell sorting (Wadajkar et al., 2014). To enhance the surface to volume ratio of the culture surface and, therefore, to enhance the total MSC yield, several researchers demonstrated the use of polymeric micro-carriers, with sizes ranging from tens to hundreds of microns in diameter, as culture substrates in contrast to the use of conventional tissue culture flasks (Chen et al., 2013). Several investigations have explored the use of biomaterials to guide the direct-differentiation or induced-differentiation of MSCs, as a means to avoid the use of several media supplements that have been shown to affect MSC characteristics and their differentiation potentials (Huang, C. et al., 2015; Lee et al., 2013; Oreffo et al., 1998; Tay et al., 2010; Wang et al., 2014). For example, it has been shown that osteogenesis is promoted when MSCs are cultured on stiff (~40 kPa) polyacrylamide hydrogel substrates whereas they can be induced into cells that express markers of neurogenesis when cultured on soft (~0.5 kPa) gels (Lee et al., 2014). Furthermore, biomaterials are being used as carriers for MSC delivery, for example by their attachment to or encapsulation -in polymeric microspheres, in order to achieve effective localization of a MSC graft to the target implantation site. This direct targeting is difficult to achieve by conventional injection of MSC suspensions in saline (Chan et al., 2007; Choi et

al., 2005; Lee et al., 2016; Russo et al., 2014). However, biomaterials influence MSC behaviour in numerous ways and it remains elusive as to how the physical, chemical and mechanical signals offered by these biomaterials are sensed and adopted by the cells. In this context, understanding the cell – materials interactions at the molecular level should offer important clues to the mechanisms involved.

3. Comparative transcriptome analysis and applications to study MSCs

3.1. Identification of biomarkers and donor variations

The majority of the MSC transcriptome work in the literature to date has been carried out by using whole genome expression microarrays or quantitative polymerase chain reaction-based assays. These investigations demonstrated the potential of transcriptome analysis in many of the MSC areas, including determination of the spectrum of gene markers (Abdallah et al., 2015; Bae et al., 2011) and in definition of gene expression dynamics during human MSC isolation and proliferation (Abu Kasim et al., 2015; Kay et al., 2015; Ren et al., 2011; Ren et al., 2013). Comparing the global gene expression of BM-MSCs with that of HSCs, human ESCs and fibroblasts suggested that the BM-MSCs, in addition to expressing several cytokines and growth factors, express large amounts of extracellular matrix genes that are distinct from the rest of the cell types under study and therefore could be potentially used as biomarkers of BM-MSC quality (Ren et al., 2011). Transcriptional profiles of AD-MSCs were compared among young, middle- and old-aged healthy volunteers; the results showed that the aging reduces proliferative capacity and multi-lineage differentiation potential of AD-MSCs (Alt et al., 2012). Gene expression modifications were observed during repeated passage of Wharton's Jelly-derived MSCs, highlighting an issue of MSC aging which relates to MSC stability and hence application safety (Gatta et al., 2013) (**Figure 3**). Transcriptome

analysis of early and late passage human BM-MSCs showed that transcriptional changes occurred before any observed changes in phenotype during human BM-MSCs senescence. Although variable among different donors, a set of 155 senescence gene markers was identified for associations of human BM-MSCs with age by using a computational approach (Ren et al., 2013). With regard to the source of derivation of the cell populations it has been shown that human MSCs originating from different organs display distinct transcriptional profiles from each other (Abu Kasim et al., 2015; Steinert et al., 2015; Strioga, M. et al., 2012).

3.2. Tracking cell differentiation

MSC differentiation has been a focus of past comparative transcriptome approaches, which has included studies of functional differences in MSC populations (Jansen et al., 2010), characterization of MSC stemness (Gao et al., 2013; Steinert et al., 2015), and assessments of differentiation status (Brown et al., 2015; Granchi et al., 2010; Liu et al., 2014; Menssen et al., 2011; Modder et al., 2012; Satija et al., 2013; Skreti et al., 2014; Steinert et al., 2015; Wislet-Gendebien et al., 2012; Yu et al., 2013; Zhang et al., 2014). For example, temporal and spatial patterns of gene expression profiles were reported for chondrogenic differentiation (Skreti et al., 2014). During adipogenic and osteogenic differentiation, various mutations in *LMNA* gene which encodes for the nuclear lamin A/C protein influenced the differentiation efficacy of BM-MSCs in a mutation-specific manner and promoted unique gene expression patterns (Malashicheva et al., 2015). Comparative transcriptome analysis revealed that the adult human brain-derived perivascular MSCs holds comparable differential potential to that of the human brain-derived NSCs and the human BM-MSCs; thus highlighting the potential of perivascular MSCs for therapeutic use similar to neural and bone-marrow stem cells (Lojewski et al., 2015).

3.3. Exploring disease association

Disease association is another application of comparative transcriptome analysis to MSC populations derived from control and pathological states (Benisch et al., 2012; Binato et al., 2015; de Oliveira et al., 2015; Huang, J.C. et al., 2015; Li et al., 2012). Remarkable abnormality in gene expression was observed in human BM-MSCs from aplastic anaemia patients when compared with those from healthy volunteers (Li et al., 2012). Transcriptome abnormalities were also observed in human BM-MSCs of acute myeloid leukaemia (Huang, J.C. et al., 2015) and multiple sclerosis patients (de Oliveira et al., 2015). A number of candidate genes relating to the leukemic transformation process were identified (Binato et al., 2015; Huang, J.C. et al., 2015). Analysis of the transcriptome and micro RNAs of breast resident AD-MSCs revealed that migrating cells displayed similar profiles to those of resident AD-MSCs in this condition, suggesting a role of resident AD-MSCs in breast cancer development (Senst et al., 2013). Human BM-MSCs transcriptomics was also used to investigate the accumulation of progerin and prelamin A in premature aging diseases (Infante et al., 2014). As increasing numbers of transcriptome datasets became available, computation data mining has been applied to investigate MSC gene expression profiles in elderly patients with osteoporosis (Zhou et al., 2015).

3.4. Optimizing culture conditions

Apart from optimizing the conventional culture conditions such as oxygen levels, medium composition and etc., understanding the role of scaffolds, the biomaterials that add the third dimension to cell culture, has now invariably become part of the process. Comparative transcriptome analysis has been employed to investigate the cell response, particularly MSCs, in such 3D culture systems (Gatta et al., 2013; Hu et al., 2014; Kim et al., 2014; Pedersen et al., 2013; Roobrouck et al., 2011; Wu et al., 2012; Han et al., 2013;

Jakobsen et al., 2014; Neuss et al., 2011). For example, a study by Han et al suggested that the 3D culture conditions enhanced the self-renewal and pluripotency of NSCs and BM-MSCs (Han et al., 2013). However, it is important to pay attention to the composition of the scaffolding biomaterial. For instance, Neuss et al demonstrated that MSCs performed better on the scaffolds made with poly(lactic acid) than those made with poly(ϵ -caprolactone) (Neuss et al., 2011). Besides, the role of differentiation factors such as TGF β should also be investigated in order to modulate the differentiation effectively. But in contrast to the traditional studies involving one factor at a time, it may be useful to perform multifactorial studies in order to understand the interdependency, if any, of such differentiation cocktail. For instance, a recent study based on high throughput mRNA-profiling of MSCs cultured on a 3D substrate in 48 different conditions of TGF β 1, 2 and 3, bone morphogenic protein 2, 4 and 6, dexamethasone, insulin-like growth factor 1, fibroblast growth factor 2 and cell seeding density suggested that a cocktail composed of TGF β and dexamethasone was the best differentiation cocktail. However, this also induced undesirable molecules, thus, indicating a need for improvement in the differentiation protocols (Jakobsen et al., 2014).

3.5. Health and safety aspects

In consideration of clinical safety using MSCs, a culture medium that is serum-free and free of all xenogeneic materials was developed. Transcriptome analysis of human BM-MSCs and AD-MSCs cultured in this medium displayed transcriptional profiles comparable to those using the traditional culture media (Chase et al., 2012). AD-MSCs derived hematopoietic cells acquired cell surface human immunodeficiency virus-receptor and co-receptors during differentiation *in vitro* and became permissive to viral infection whereas undifferentiated AD-MSCs did not show productive infection (Nazari-Shafti et al., 2011). Human BM-MSCs may undergo spontaneous changes during long-term culture and immunosuppressive and anti-inflammatory properties may be lost (Infante et al., 2014).

Studies comparing FL- and PL- MSCs with that of late or post pregnancy MSCs have typically ignored the gestational age; according to a recent report, this is not safe or accurate mainly because the fetus and the placenta grow rapidly with divergent developmental requirements during the first trimester (Ryan et al., 2014).

4. NGS: technology advantage and applications in MSC research

4.1. Evolution of gene sequencing technologies

Gene sequencing began with two-dimensional chromatography in the 1970s but gained momentum with the introduction of a more reliable and reproducible Sanger chain termination method in 1977 (Anonymous, 2016; Goodwin et al., 2016). Subsequently, the capillary electrophoresis based platforms were introduced during the 1980s and 90s as the “first-generation” sequencing technologies (Anonymous, 2016; Goodwin et al., 2016). Considered as the high-throughput technologies of that time, they were popular for their general use in the Human Genome Project. However, the first truly high-throughput sequencing platform, the Genome Analyzer, introduced in 2005, enhanced the data output from 84 kilo bytes to 1 giga bytes per run and paved the way for the “next-generation” sequencing technologies (Anonymous, 2016; Goodwin et al., 2016). Within the past decade, several NGS platforms have been developed which decreased the genomic sequencing time from years to a few hours and the cost from billions of US dollars to just 1000 dollars each while increasing the data output to 1.8 Tb per run (Anonymous, 2016; Goodwin et al., 2016). Furthermore, platforms have been developed to sequence the protein-interacting DNA fragments as well as the RNA, and therefore extend the applications of NGS technologies from genomics to epi-genomics and transcriptomics; which collectively revolutionized the fields of molecular biology and the omics beyond imagination. Detailed information on NGS

was recently reviewed by Goodwin et al. and consequently is not addressed further in the present review (Goodwin et al., 2016). However, an overview of the various NGS technologies and the relevant commercial platforms with their capabilities in terms of throughput and run time is presented in Table 1.

4.2. NGS in transcriptomics research

An array of NGS-based methods such as total RNA-seq, mRNA-seq, small RNA-seq and targeted or pathway-oriented RNA-seq was developed to get a comprehensive view of the transcriptional profile of a cell at a given time. The total RNA-seq involves the conversion into cDNA before proceeding for NGS processing (**Figure 4**), while, other RNA-seq includes additional enrichment steps before being converted to cDNA and subsequent NGS processing. In any case, a typical NGS transcriptome run produces giga bytes of sequencing data that normally covers tens of thousands of genes. The detected sequence reads are subject to quality control pipelines and the high-quality reads are mapped to genome reference databases for gene identification and annotation. Unmapped high-quality reads are often novel gene transcripts or products of pathogen origins (Gao et al., 2014). Using the reads of annotated genes, the expression level of each gene can be normalised as RPKM or FPKM. Then quantitative comparisons can be made between samples to detect DEG. Using the DEGs, protein-protein interaction networks and gene function pathways down- and up-regulated by a treatment can be identified, facilitating constructions of the gene regulation and interaction networks for making functional interpretations. NGS has advantages over microarray technology because the latter can only detect genes that have specific probes printed on the microarray which is limited in the number of target genes and often incapable of detecting novel gene products (Zhao et al., 2014) including small RNA such as micro RNA, noncoding RNA and small nuclear RNA.

4.3. NGS-based MSC transcriptomics

Although it has been a decade since the first NGS platform was introduced, NGS technologies were mainly explored for whole genome sequencing projects as shown by the number of publications. However, application of NGS technologies in whole transcriptomics analyses has gained over the past 6 years. However, the use of these platforms in the MSCs research area has not been explored fully. Hence, to emphasise the potential value of this technology in this field we summarize recent progress here to demonstrate the remarkable power and potentials of NGS.

4.3.1 NGS in understanding MSC biology

Aiming to characterise two closely related stromal cell types, AD-MSCs and dermal fibroblasts, and to analyse the dynamics of their *in vitro* differentiation processes, multiplex mRNA-sequencing was performed using the Illumina Genome Analyzer IIx deep sequencing system (Jaager et al., 2012). Forty-five million annotated reads were obtained and 60% of them were mapped to known human transcripts. Global transcriptome profiling revealed specific gene expression patterns of AD-MSC and dermal fibroblasts, demonstrating that the two cell types were distinctly different at the undifferentiated stage. During *in vitro* differentiation, the cell-specific patterns were maintained and notable lineage-specific gene regulation occurred in the early stages of differentiation which persisted over time. Despite the difference, AD-MSC and dermal fibroblasts underwent a similar process in adipogenic and osteogenic differentiation *in vitro* but displayed distinguishable dynamics of chondrogenic differentiation (Jaager et al., 2012).

Cultured human BM-MSCs and PL-MSCs were compared for their transcriptomes using the Illumina GAIIX system (Roson-Burgo et al., 2014) (**Figure 5**). From 5,271 detected protein coding genes, 203 DEGs were detected between the two MSC lines. Gene ontology

enrichment indicated that the BM-MSC overexpressed genes supporting functions of bone biogenesis, bone formation, blood vessel morphogenesis, extracellular matrix organization and inflammatory response. PL-MSC, however, displayed up-regulated gene expression relevant to embryonic morphogenesis, cell cycle activation and negative regulation of cell death. The authors interpreted that such difference reflected the biological properties of the two MSC subtypes, i.e., the bone and fetal origins. A protein-protein interaction network was constructed for the mesenchymal regulatory transcription factors. Cell subtype specific isoforms of MSCs markers and transcription factors were detected due to alternative RNA splicing (Roson-Burgo et al., 2014).

4.3.2 NGS and MSCs in understanding developmental biology

In an integrated investigation on epigenetic mechanisms of embryo development, hESC were differentiated to mesendoderm cells, neural progenitor cells, trophoblast-like cells and MSCs for characterizations of DNA methylation, chromatin modifications, and the transcriptome in each lineage (Xie et al., 2013). Using the Illumina HiSeq2000 system, expressions of 76.6% of the 19,056 known human coding genes were detected. In addition, transcripts for 2,175 known and 281 novel lncRNA genes were determined in at least one cell lineage. Among them, 37.9% of total expressed lncRNA genes were lineage-restricted whereas only 16.5% of expressed coding genes exhibited lineage restriction. Furthermore, significant percentages of transcription start sites of lncRNA genes directly fell into the long terminal repeat-containing retrotransposons and this percentage was notably high (31%) in MSC-enriched lncRNA genes (Xie et al., 2013).

4.3.3 NGS and MSCs in understanding disease conditions

To investigate the mechanism that MSCs may enhance tumour progression, transcriptomes of TGF α stimulated BM-MSCs were analysed using the SOLiD 5500xl

platform (De Luca et al., 2014). Sequence reads of 37 million for MSCs and 47 million for MSCs+TGF α were obtained. When aligned with the human genome reference, majority of the reads (92%) mapped to exons, whereas the rest mapped to introns (4%) or intergenic regions (4%). Between the two samples, 1,640 DEGs, including many secreted proteins and cell surface receptors, were detected. The difference on RNA alternative splicing was also observed and a novel isoform of the secreted vascular endothelial growth factor protein of 172 amino acids was discovered only in the TGF α treated MSCs (De Luca et al., 2014).

With interest in the molecular mechanisms of neoplastic transformation in human cells, a human MSC line UE6E7T-3 was immortalized by using human telomerase reverse transcriptase and human papillomavirus type 16 E6/E7 genes. The immortalized cell line lost a copy of chromosome 13 and underwent pro-tumorigenic changes in prolonged culture which displayed tumorigenicity in nude mice. Comparative transcriptome analysis was performed using the SOLiD 3 PLUS System (Takeuchi et al., 2015). Among 8,032 genes, in which expression levels significantly changed during the neoplastic transformation, 1,732 genes were selected for further analysis based on pathways and cellular functions. Overexpression of oncogenes, together with genes related to anti-apoptotic functions, cell-cycle progression, and chromosome instability were observed, as well as decreased expressions of tumour suppressor genes. At the later stage, overexpression of glypican-5 (*GPC5*) which encodes a cell-surface proteoglycan and Patched (*Ptc1*), the cell surface receptor for hedgehog (*Hh*) signalling, were detected and co-localized, suggesting function associated with the neoplastic transformation (Takeuchi et al., 2015).

To understand the disease mechanism of the paediatric myelodysplastic syndrome (MDS), BM-MSCs from paediatric MDS patients were analysed using Illumina DeepSAGE sequencing (Calkoen et al., 2015). Different to the MSC gene expression abnormality reported in adult MDS, BM-MSCs of paediatric MDS displayed gene expression alternations

associated with cell survival and malignant transformation. The authors concluded that the observations supported the notion that paediatric MDS and adult MDS are two different diseases (Calkoen et al., 2015).

4.3.4 NGS in MSC-based regenerative medicine and tissue engineering

To investigate the potential of MSCs in regenerative medicine applications, a transcriptome level study was performed with an *in vitro* co-culture model of MSCs and vascular endothelial cells (Li et al., 2015). The Illumina HiSeq2000 system was used to produce >36 million raw reads for each sample. Approximately 80% of the reads were mapped to >16,000 human genes. The number of DEGs increased during the culture course between monocultures and MSC-endothelial co-cultures. Expression of angiogenic genes were induced and smooth muscle commitment was observed in co-culture. Validated by NF- κ B signalling activation experiments, the authors demonstrated that MSCs secreted, in a contact-dependent manner, two interleukins (IL1 β and IL6) that promoted the angiogenesis-related gene activation in human umbilical vein endothelial cells during co-culture (Li et al., 2015).

Similarly, in another study on MSCs based renal tissue engineering, Ion Torrent NGS was used to investigate gene expression dynamics during *in vitro* investigations of human BM-MSC reprogramming into functional renal proximal tubular-like epithelial cells (RPTEC) induced by HK2 (an RPTEC cell) based cell-free extracts (Papadimou et al., 2015). Inter-group comparisons using scatterplots and Pearson coefficient correlations showed that CL17 cells (the reprogrammed human BM-MSCs) exhibited gene expression patterns more closely to HK2 cells than to native BM-MSCs. Far more DEGs, which imply greater differential response, were detected between CL17 cells and BM-MSCs (2,127 up- and 2,355 down-regulated DEGs) than between CL17 and HK2 cells (120 up- and 61 down-regulated

DEGs). Further, function enrichment of DEGs revealed that the *EGFR* pathway was up-regulated in the MSC-RPTEC reprogramming (Papadimou et al., 2015).

However, because the cell – biomaterials cross-talk often depends on the stemness of MSCs, a careful consideration of MSCs lineage signature is required for cell therapy applications. As evident from a recent study on AD-, PL- and BM-MSCs, 489 genes were identified based on a deep comparative analysis of multiple transcriptomic expression data series including MSCs of different tissue origins, MSCs in different states of commitment and other related non-MSCs cell types such as dermal fibroblasts (Roson-Burgo et al., 2016). Such information is perhaps helpful to pick up MSCs in a right stage of lineage commitment for cell therapy applications. While it is essential to understand MSCs – biomaterials interactions, it is also equally important to understand how physiological mechanisms and regulatory processes of MSC trafficking to the injured tissue. To this end, RNA sequencing was used to study the gene expression profile and migratory potential of hMSCs and it was found that the chemotaxis of TLR4-primed hMSCs was orchestrated by IRF1 and NF- κ B (Kim et al., 2016).

5. Concluding remarks and future directions

Comparative analyses of transcriptomes permits the identification of genes that are differentially expressed in discrete cell populations or in reaction to various treatments. RNA analysis was initially limited to tracking individual transcripts by Northern blots or quantitative PCR, but with the advent of microarray and NGS technologies, high throughput transcriptomics became possible. While microarrays detect only known sequences and suffers from background hybridization and probe saturation, NGS has dramatically advanced the field of transcriptomics as it can virtually detect any RNA type. Understanding global gene expression profiles with the use of such technologies is highly relevant to research on stem

cell biology both from a fundamental and a translational perspective. In particular, MSCs with their self-renewal, multi-potent and immune-modulatory abilities have emerged as a potential candidate for cell therapy applications. Furthermore, in combination with evolving interdisciplinary technologies such as gene therapy, biomaterials, and immune-regulatory therapies the horizon for the potential clinical uses for MSCs is continuously expanding.

Yet, several issues such as isolation with high accuracy and yield, *in vitro* expansion with maintenance of stemness characteristics and controlled differentiation protocols in 2D and 3D cultures needs to be addressed. Several investigations have explored both microarray and NGS technologies to perform the transcriptome of MSCs in order to characterize the cells at the molecule level in different contexts, such as investigating the source dependent, culture conditions dependent, etc., differences in MSCs. In the past five years, benefitting from the technological advances and cost reduction of NGS platforms, it is clear that transcriptome analysis is being increasingly deployed in MSCs research. Yet, surprisingly, the number of investigations dealing with the transcriptomics of MSCs are relatively low when compared to the total of transcriptomics investigations reported in the literature. Based on the successful examples briefly described in the present report, we identify a few areas, as follows, where application of transcriptomics in MSC research would be opportune.

From the fundamental research perspective, a) detailed investigations on the global transcriptome of MSCs may lead to the discovery of new human transcripts both coding and noncoding genes; this would not only further our understanding of the gene activity in MSCs but also shed light on the role of noncoding genes in the MSCs function; b) investigations on the global transcriptome analysis focused on the mitochondrial genes may offer important clues into the interplay between mitochondrial and nuclear genomes and the role of mitochondrial gene sets on properties of MSCs; and, c) global gene expression analysis may

also be focused on identifying non-human reads in order to investigate the elements from non-human origin such as viruses.

From the translational research perspective, a) efforts on the comparative transcriptomics analysis of MSCs from different tissue sources are needed to identify tissue-specific surface markers; b) investigations focused on the comparative studies of MSCs from different donors, differing in gender and age, for example, may offer clues from the population genetics perspective; c) efforts on understanding the molecular phenotype of MSCs with respect to the culture conditions such as serum vs serum-free, hypoxia vs normoxia and other biological factors are needed to optimize the conditions in order to achieve the optimal expansion of MSCs *in vitro* with high yield and quality for clinical therapeutic use; d) as a means to get an accurate record of the quality control, investigations on contamination checks necessary during culture may be of great value, and e) understanding the interactions of MSCs with biomaterials at the molecular level is essential to achieve controlled differentiation.

However, when it comes to *in vitro* experimentation, we envisage inconsistencies and issues with reproducibility in this exciting area, not due to the errors on part of the researchers or the sequencing platforms, but purely owing to the fact that the MSCs population is quite heterogeneous. Collaborative efforts may be needed to develop standard operating procedures for isolation, purification and expansion. Possibly, such efforts may substantially minimize the anticipated batch to batch variation, but, given the fact that the cells in a given population may still be in different phases of proliferation or differentiation, they may still lead to discrepancies in the expression profile. One possible solution may be to synchronise the cell population before performing any investigations; however, this needs to be proved experimentally.

We believe that it is also of significant interest to compare the *in vitro* observations, be in 2D or 3D cultures conditions, with that of the *in vivo* results. A recent study of this kind shows that such efforts will allow us to understand better and help to recapitulate the native microenvironment of cells of interest (Yeung et al., 2015). In addition, there have been exciting recent advances in the determination of the genomic and transcriptomic landscapes of single cells in the field of spatially resolved omics (Crosetto et al., 2015). In the future, such studies will lead eventually to the determination of the degrees of correlation between observed *in vitro* and *in vivo* physiological mechanisms. Defining these different environmental impacts on the mechanisms of cell activation, differentiation and the potentials for lineage commitment in normal and pathophysiological states will help clarify the value of MSCs for therapeutic use in regenerative medicine.

In conclusion, scientific discovery is driven in large part by technological advances (Balaram, 2002) and it is highly likely that increasing application of NGS technology in transcriptomics will yield dramatic new discoveries in the MSC field in the near future.

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Conflict of interest

The authors and the sponsor declared no conflict of interest.

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Figure legends

Figure 1. MSCs potential and bottlenecks: Discovery of new tissue sources and development of defined protocols for easy isolation, culture and differentiation into lineage- and non-lineage- cell types, the discovery of immune-modulatory ability, convergence with gene therapy and biomaterials technologies opens up the immense potential for MSCs both from a fundamental as well as a translational perspective. However, isolation with high accuracy and yield, *in vitro* expansion without losing stemness and efficient differentiation protocols for two-dimensional as well as three-dimensional cultures need to be addressed not only at the cellular level but also at the molecular level.

Figure 2. An outline of microarray or gene chip technology: The isolated RNA is converted into cDNA and labelled with fluorescent dyes such as Cy-3 and Cy-5 to make target sequences. Equal amounts of test and control target sequences are mixed and are subjected to hybridization with the gene-specific probes, immobilized in a discrete location with spot size as small as 50 μm , of a microarray or gene chip. The gene is scanned using an imaging platform and the signal intensity in a given spot corresponding to a given gene is used to determine the number of copies.

Figure 3. An example of microarray-based transcriptome data: Hierarchical clustering analysis suggested that *in vitro* expansion of Wharton's Jelly-derived MSCs up to 12 passages induce up-regulation of 157 genes (cluster 1) and down-regulation of 440 genes in comparison to the 4th passage (cluster 2). In the figure, all the related genes are clubbed as per their expression values, expressed as log ratios. Each row represents one gene, each column the 6 different runs. The quantitative variations in gene expression across all the samples are denoted in different colours: over-expressed genes in red, down-regulated genes in green, no change in black, and missing data points in grey. The top labels show the different

experiments. Reproduced from (Gatta et al., 2013). ©Gatta et al.; licensee BioMed Central Ltd. 2013.

Figure 4. An outline of a next-generation sequencing approach: The isolated RNA is converted into cDNA, fragmented into pieces and ligated with adapters to create templates. The templates are then hybridized with the substrate-bound complementary adapters. These adapters act as primers and, following a polymerase chain reaction using a DNA polymerase and fluorescently labelled dNTPs, aid in the synthesis of the second strand. The incorporation of a nucleotide during such synthesis at a given location is identified by the fluorophore excitation and accordingly converted into sequencing data. Reads of test and control samples are then aligned to a reference sequence so as to identify DEGs. This approach is called *sequencing by synthesis*, however, there are various other NGS platforms; for more details please refer to Table 1.

Figure 5. An example of NGS-based transcriptome data: RNA deep sequencing of human MSCs derived from two different sources (bone marrow – BM and placenta – PL) recorded the genome-wide expression profile of 5,271 protein-coding genes and identified 203 source-related DEGs that translate into about 5% distance between BM- and PL- MSCs lineages. (A) Scatter plot presenting the values of $\log_2 (FPKM_{sum})$ for each gene in the BM- MSCs (X-axis) vs the PL- MSCs (Y-axis). (B) Scatter plot including the $\log_2 (FPKM_{means})$ of the protein-coding genes in the BM- and PL- MSCs (showing with a green shade the region that includes 95% of the expressed data distribution). (C) Table indicating the $\log_2 (FPKM_{sum})$ values corresponding to 8 marker genes in the BM- and PL- MSCs. (D) Phase contrast micrographs of human PL- and BM- MSCs in the culture at passage 3. Reproduced from (Roson-Burgo et al., 2014). ©Roson-Burgo et al.; licensee BioMed Central Ltd. 2014.

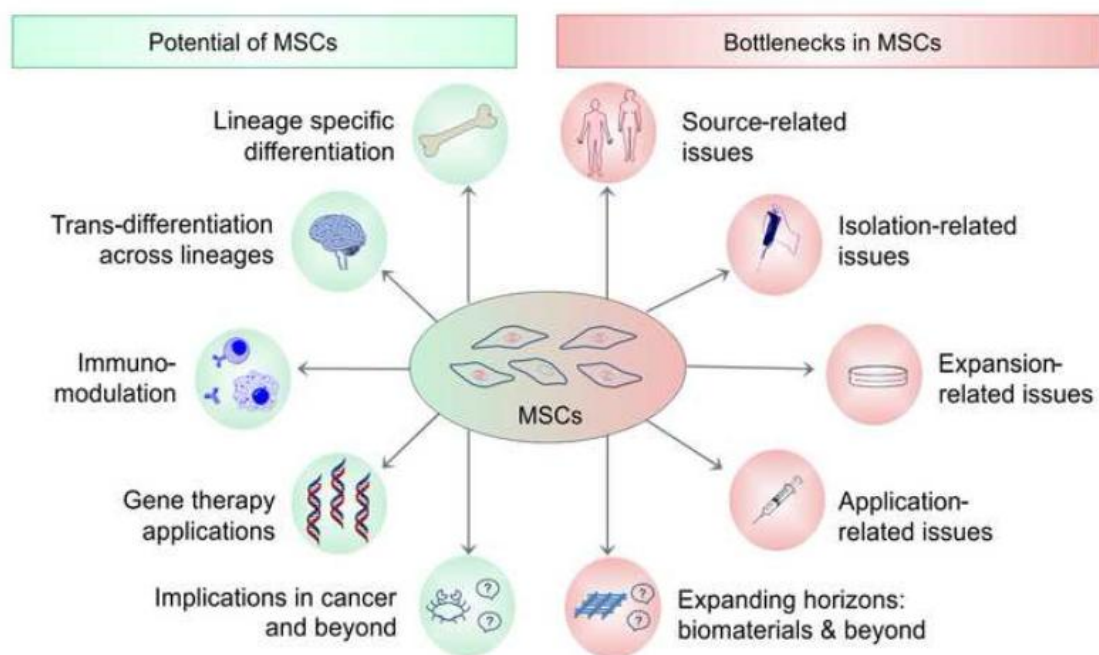


Figure 1

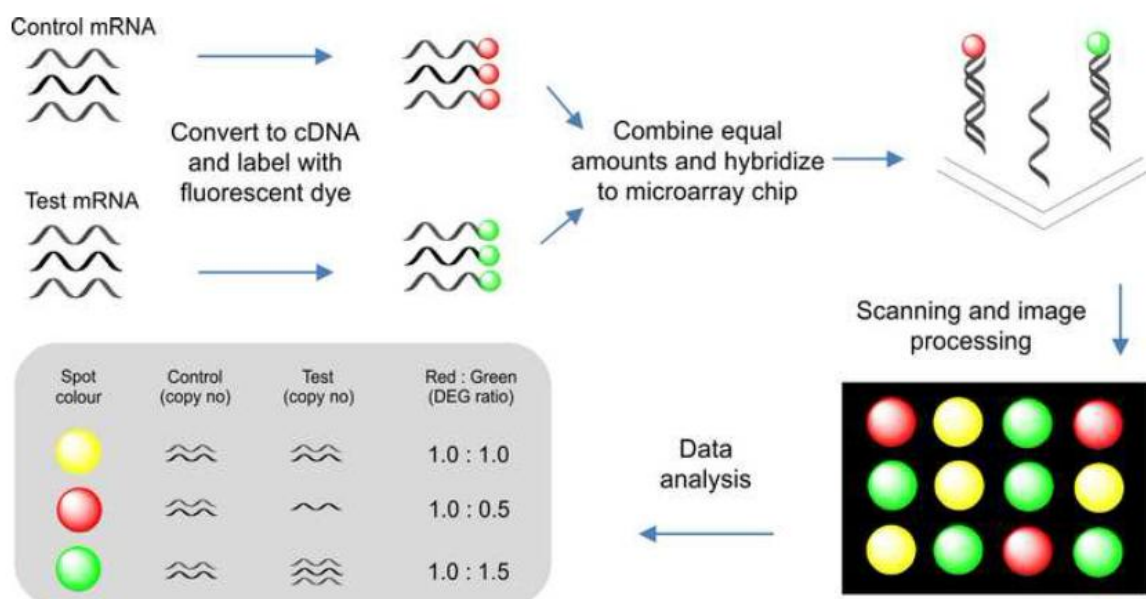


Figure 2

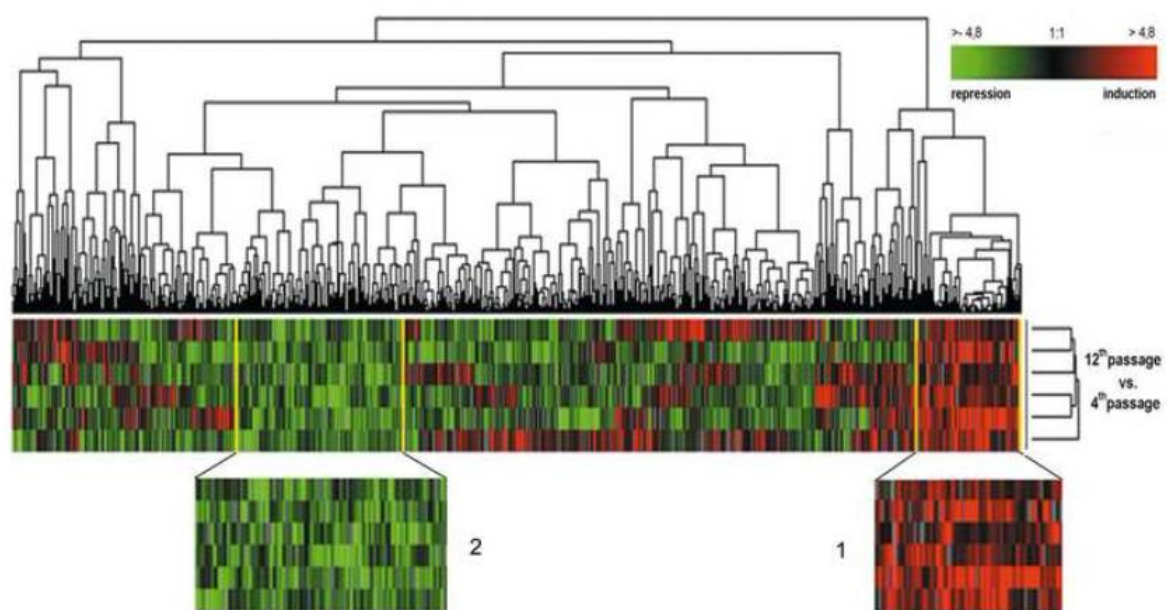


Figure 3

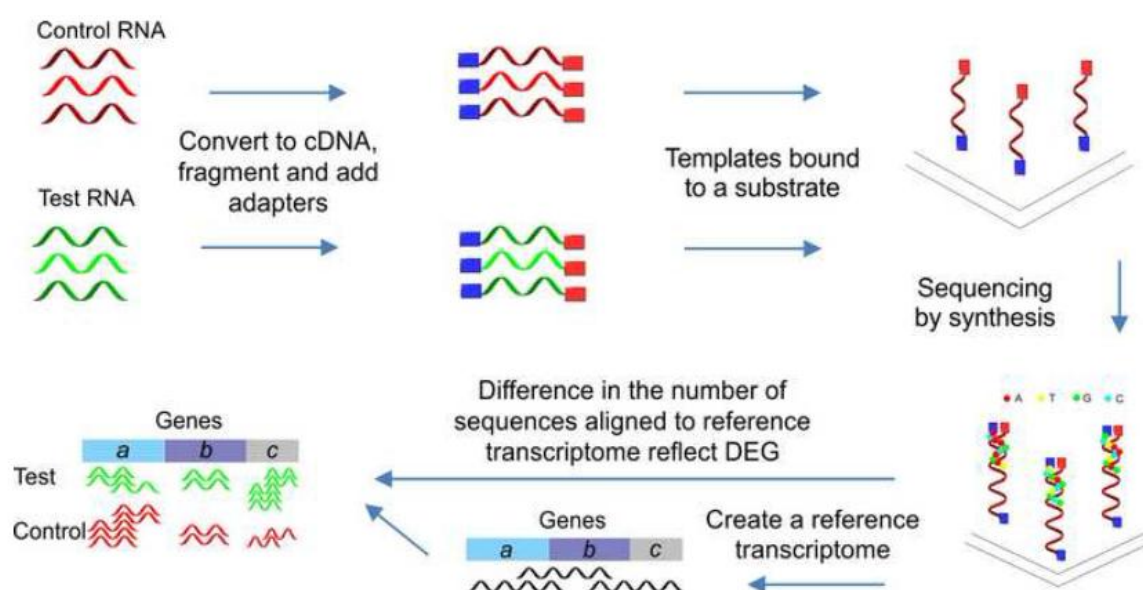


Figure 4

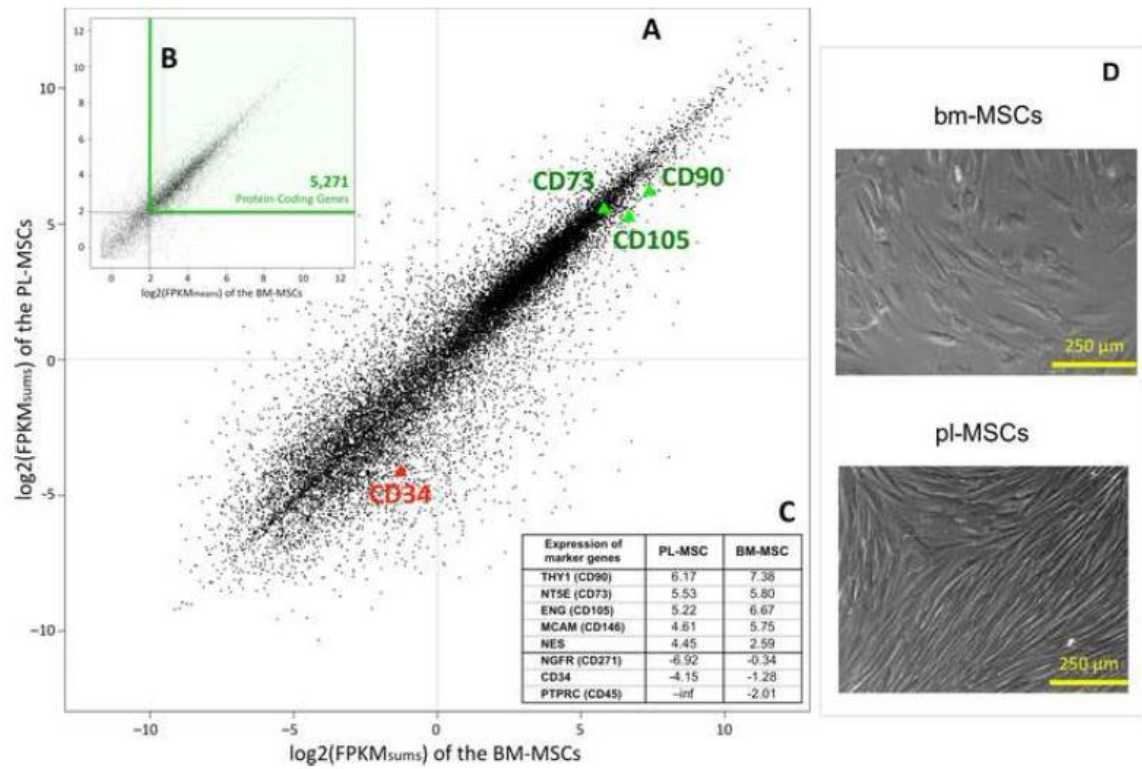


Figure 5

Table 1

An overview of various NGS technologies and relevant commercial platforms with few remarks on their range of capabilities.

Name of the technology	Details of the technology	Commercial platforms developed ^a	Remarks ^a
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Short-read sequencing approaches

Sequencing by ligation	It involves “hybridization” of <i>probes</i> (oligonucleotide strands labelled differently with fluorescence dyes) and <i>anchors</i> (short oligonucleotide strands of known sequence) with the <i>target</i> DNA strand which was flanked with a known <i>adapter</i> strand; wherein, <i>probes</i> bind to <i>target</i> and <i>anchors</i> bind to <i>adapters</i> respectively. Followed by “ligation” of <i>probe</i> and <i>anchor</i> when its bases (at least a few) match with the <i>target</i> , in presence of a <i>DNA ligase</i> . Based on the fluorescence produced by the <i>probe</i> , one can infer the identity of the nucleotide at this position in the unknown sequence.	SOLiD (Wildfire, BGISEQ-500 (FCS155, FCL155)	5500 xl), pairs throughput, and 24 h to 6 d run time.
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Sequencing by synthesis (SBS): cyclic	It involves the use of <i>DNA polymerase</i> , a mixture of <i>dNTPs</i> and <i>primers</i> (complementary to <i>adapter</i> sequence flanked to <i>target</i> sequence) to carry out the	Illumina MiniSeq (Mid output, High output), Illumina MiSeq (v2, v3), Illumina NextSeq	540 megabase pairs to 900 gigabase pairs throughput, and 4 h to 11 d run
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reversible synthesis of a new strand that (500/550 Mid output, time; except
 termination would be complementary to the 500/550 High output), Qiagen
 (CRT) *target* sequence, and therefore is Illumina HiSeq2500 GeneReader
 comparable to a typical PCR. But, (v2 Rapid run, v3, which is capable
 unlike PCR, the four *dNTPs* are v4), Illumina of detecting 12
 differently labelled with a HiSeq3000/4000, genes with up to
 fluorophore and are modified to Illumina HiSeq X, 1,250 mutations
 have a *terminator* molecule Qiagen GeneReader. and takes up
 blocking 3'-OH group; Therefore, several days of
 during each cycle, elongation run time.
 takes place by addition of just one
 nucleotide. By imaging the
 fluorescence signal at each cycle,
 one can decipher the sequence of
target DNA.

Sequencing Fundamentally, it is similar to 454 GS (Junior, 30 megabase
 by CRT based sequencing approach. Junior+, FLX pairs to 15
 synthesis: But, unlike CRT, SNA uses four Titanium XLR70, gigabase pairs
 single- *dNTPs* differently labelled with a FLX Titanium XL+), throughput, and 2
 nucleotide fluorophore but without a Ion PGM (314, 316, h to 23 h run
 addition *terminator* molecule, and, the 318), Ion Proton, Ion time.
 (SNA) reaction mixture contains only one S5 (520, 530, 540).
 of four *dNTPs* at a time. As a
 result, each of the four nucleotides
 must be added alternatively to a
 sequencing reaction. The absence
 of next nucleotide in the reaction
 mixture prevents elongation, thus,
 the elongation proceeds at a rate
 of one nucleotide per cycle.
 Subsequently, imaging the
 fluorescence signal at each cycle
 allows decoding the *target* DNA

sequence.

Long-read sequencing approaches

Single-molecule real-time long reads	PacBio systems are fundamentally similar to SBS involving a DNA polymerase to synthesize a new strand and track addition of a nucleotide based on the respective fluorescence signal; but, unlike SBS, PacBio systems immobilize <i>DNA polymerase</i> instead of a <i>template</i> . While Oxford Nanopore systems are straightforward and, unlike other systems, they neither require any hybridization, elongation or ligation reactions nor involve secondary signal detection; they use a specially made protein pore to allow a <i>target</i> DNA to pass-through and while doing so detects the current shifts which subsequently translated into sequencing data.	Pacific BioSciences 500 megabase (RS II, Sequel), pairs to 4 terabase Oxford Nanopore pairs throughput, (MK 1 MinION, and 0.5 h to 48 h; PromethION) except Oxford Nanopore PromethION for which run time data is not available at the moment.
Synthetic long reads	Synthetic long read approaches do not perform any long read sequencing process in a true sense. Instead, they involve the assignment of barcodes to sheared fractions of a long <i>target</i> DNA, followed by the sequencing of these fractions with the help of the existing short read platforms, and finally, the assembly of these	Illumina Synthetic Throughput and Long-Read, 10X run time comparable to HiSeq2500 platform

short reads into a long read with the help of computational tools. Further, in a way, segregation of the sheared fractions allow the isolation and local assembly of sequences that are otherwise complicated or unresolved by other approaches.

^a Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Genetics (Goodwin et al., 2016), copyright 2016.

Highlights

- Mesenchymal stromal cells hold immense potential for cell-based therapies & beyond
- However, the molecular fingerprint of MSCs in such contexts still remains elusive
- Transcriptomics studies based on microarray proved valuable in MSC biology
- Next-generation sequencing offers affordable and robust transcriptomics platforms
- NGS can advance the MSC biology both from fundamental & translational perspective