Quantitative correlations among human mesenchymal stem cell mechanical properties and biological function

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Abstract

Mesenchymal stem cells (MSCs) are derived from bone marrow, and are capable of proliferating and differentiating along multiple pathways such as osteoblasts, chondrocytes and adipocytes. MSCs offer the means for regenerative therapies not possible with conventional small molecule/antibody/ nucleic acid therapeutics. However, all MSCs are not equivalent. Adult MSCs (aMSCs) derived from infant or adult sources are heterogeneous, exhibit poor overall integration in host tissues, and their differentiation and proliferation capacities are limited by *ex vivo* culture. On the other hand, fetal MSCs (fMSCs) derived from fetuses are more homogeneous, plastic and grow faster than aMSCs. However, they face serious ethical and practical issues that limit their applications. For these reasons, we hypothesized that aMSC populations contain a subpopulation with similar biophysical and biological properties to fMSCs. To verify this thesis, we studied aMSC size distribution, aMSC migration velocity and aMSC mechanical properties. We explain later in this work why we chose these characteristics. We were then able to find a subpopulation of aMSCs with similar size distribution to fMSCs. We were not able to find a subpopulation of aMSCs with similar migration velocity to fMSCs. At last, we were able to prove the existence of a subpopulation of aMSCs with similar mechanical properties to fMSCs.

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Chapter 1. Introduction

1.1. Mesenchymal stem cells and clinical applications

The bone marrow stroma creates a unique microenvironment that regulates proliferation, differentiation and maturation of mesenchymal stem cells (referred later as MSCs). The bone marrow is composed of well-documented elements including reticular cells, osteoblasts, endothelial cells, smooth muscles and macrophages. Stem cells within the bone marrow stroma were described first twenty years ago, and were mostly studied for their hematopoietic counterparts [1]. Caplan introduced the term "mesenchymal stem cells" in 1991 [2] and eight years later, MSCs were demonstrated multipotent *in vitro* [3]. This differentiation capacity and the potential applications of MSCs for human diseases treatment made this research one of the most intensely investigated cell-based therapy options over the last decade.

1.1.1. Isolation and characterization of mesenchymal stem cells

Friedenstein *et al.* demonstrated in 1970 that some bone marrow cells were adhering to plastic and forming colonies *in vitro* [4]. MSCs are still derived from bone marrow using this colony forming unit-fibroblast (CFU-F) assay. However, this protocol has been improved by using density centrifugation [3] [5]. After plating in "MSC" medium, the cells are spindle-shaped and multiply rapidly to reach full confluence in around 14 days. Even if MSCs were first found in bone marrow, we now know them to be present in placenta [6], trabecular bone [7], lung [8], adipose tissue [9], synovium [10], skeletal muscle [11], periosteum [12], heart [13], teeth [14], amniotic fluid [15], and umbilical cord blood [16]. In this work, we used two types of MSCs

both coming from human bone marrow: "commercially available" adult MSCs (referred to hereafter as aMSCs, with patient sources specified later) and fetal MSCs (referred to hereafter as fMSCs) isolated from femur bone marrow of fetuses.

Despite the standardized method to isolate MSCs, it is obvious that this cell population is heterogeneous in size, proliferative capacities and differentiation potentials [17]. Some researchers have also tried to isolate homogenous populations of MSCs studying specific cell-surface antigen markers. For now, MSCs are defined by the absence of haematopoietic and endothelial surface markers including CD11b, CD14, CD31, CD34 and CD45 and the high expression of nonspecific markers including CD73, CD90, and CD105 [18]. Although these markers are present/absent in all MSCs, they are also present/absent in other cells and it is therefore required to study cell capacity for trilineage differentiation to uniquely identify MSCs [18].

Other biochemical marker-based methods have been investigated to isolate MSCs. Gronthos *et al.* used magnetic selection of cells expressing the cell-surface markers Stro-1 and CD106 to achieve better isolation of MSCs [19]. This method gave better results in isolation of MSCs with higher differentiation and proliferation abilities. In addition, Gang *et al.* found that MSCs expressing SSEA4 could have higher differentiation potential than unsorted MSCs [20]. A portion of this thesis, along with other work in the Van Vliet laboratory, considers the alternative use of biophysical rather than biochemical markers for MSCs.

On top of these cell morphology and surface marker expression profiles, the gold standard for MSCs characterization remains functional studies. Indeed, differentiating into fat, bone and cartilage

is one key characteristic of MSC identity [21] [22] [23]. These studies often show that most MSCs can only differentiate into one or two pathways within a given laboratory. In addition, they generally demonstrate that the number of MSCs capable of multipotency decreases with passaging and that most MSCs commit to only one differentiation pathway. The pathway is most often osteogenesis and can be induced using specific medium [24]. Some MSCs still undergo adipogenesis if grown using media containing isobutylmethylxanthine [5]. Very few of those MSCs undergo chondrogenic differentiation [5]. In addition to these standard differentiation pathways, MSCs can be induced to differentiate *in vitro* to show characteristics of cardiomyocytes [25], endothelial cells [26] [27] and neural cells [28]. Although Rose *et al.* showed that MSCs co-cultured with cardiomyocytes express cardiac specific genes, those cells exhibited the electorphysiological properties of stem cells and not of cardiomyocytes [29]; much work will be needed to consider those alternative differentiation pathways to be considered as defining properties [30] [31] [32].

Therefore, the present literature shows that is the putative MSC population *in vitro* is heterogeneous, including some multipotent cells and more committed cells with restricted differentiation potential. The *in vivo* behavior of MSCs remains poorly understood and studied. *In vivo* studies are difficult to perform, as intravenous delivery of MSCs result in the majority of cells trapped in the pulmonary system [33] and the number of cells reaching the target tissue is insufficient to study the effects seen on tissue function [34] [35]. One of the only ways to study MSCs *in vivo* involves scaffolds that are implanted into the patient. Kuznetsov *et al.* proved the concept implanting fibronectin-coated scaffolds seeded with MSCs into immune deficient mice [36]. This study showed some evidence that the cells migrate to

areas of tissue injury and differentiate *in vivo*. In addition to this study, it has also been increasingly recognized that the implantation of MSCs help recover from myocardial infarction through paracrine mechanisms that improve angiogenesis, decrease inflammation and support proliferation of endogenous cardiac stem cells [37] [38] [39]. Another concern emerges from implanting MSCs: that, as has been shown for embryonic stem cells that can result in teratomas *in vivo* [40], the MSCs might also differentiate into undesirable cells types and have negative physiological effects. Breitbach *et al.* showed that injection of MSCs induces risk of calcification in the hearts of animals after myocardial infarction [41]. More studies are required to confirm this finding and they will need sophisticated imaging techniques to track implanted cells and assess changes in treated patients. To understand the effects of MSCs in therapy, we will first need to properly identify and isolate MSCs. Indeed, that need for improved identification and isolation of true mesenchymal stem cells is the motivation for this entire thesis. For now, we agree that MSCs are minimally defined by plastic adherence, specific cell-surface marker profiles that are still debated among laboratories as to the exact antigens [42] [43], and *in vitro* differentiation capacities [18].

1.1.2. Mesenchymal stem cell proliferation and differentiation potentials

As mentioned in §1.1.1, researchers have reached a tacit agreement on the definition of MSCs *in vitro*. However, they still use different protocols to obtain MSCs. As clinical applications requires huge numbers of MSCs, Sotiropolou *et al.* investigated the effects of different tissue culture plastic, growth factors, basal media, glucose concentration, glutamine and plating density on MSC proliferation

[44]. Sotiropolou showed that low glucose conditions were ideal for MSC proliferation, and that all variables cited above influenced results. Sekiya *et al.* demonstrated that low plating densities increased the proliferative capacity, even if the absolute number of cells generated is decreased [45]. It is therefore important for clinical applications to find a compromise between culture conditions in order to reach high number of cells while keeping the population rich in multipotent MSCs.

As for any other mammalian tissue cell type, MSCs require blood serum for proliferation *in vitro*; different serum compositions also have different effects on MSCs proliferation and differentiation ability. That is why most researchers select batches of serum to be consistent in their experiments [21]. Serum-free culture conditions, therefore, are considered one of the keys to limit MSC heterogeneity. Pochampally *et al.* showed that serum-free culture conditions led MSCs expressing Oct-4 to have much longer telomeres than MSCs cultured with serum [46]. However, those MSCs cultured with serum-free medium stop dividing after around ten population doublings. Therefore, serum probably plays a critical role in long-term proliferation of MSCs and we have not been able to successfully culture cells in serum free conditions for now. In addition, MSCs cultured with either autologous human serum or fetal bovine serum proliferated the same way [47]. However, autologous human serum is limited in quantity and seems insufficient for long-term culture protocols and/or clinical applications. Moreover, ambient oxygen levels and specific growth factors can influence the proliferative capacity of MSCs. For example, Grayson *et al.* showed that MSCs cultured in low oxygen conditions were enriched in pluripotent cells and were better at forming bone *in vitro* and *in vivo* [48]. Growth factors also influence greatly MSC proliferation. Bianchi *et al.*

demonstrated that media with fibroblast growth factor-2 (FGF-2) helped MSCs achieving a greater total number of population doublings, and displaying longer telomeres than MSCs cultured with "standard" media [49]. Indeed, it has been shown that FGF-2 plays an important role in retarding the onset of senescence and maintaining MSC proliferation [50].

Another important factor influencing MSC proliferation and differentiation capacities is the donor characteristics, especially donor age. Stenderup *et al.* and Baxter *et al.* both showed that MSCs from "young" donors proliferate twice as much as MSCs from "older" patients [51] [52]. Guillot *et al.* also demonstrated that fMSCs proliferate at higher rates *in vitro* than do aMSCs [53]. This can likely be explained by fMSCs expressing higher expression of genes associated with proliferation and DNA reparation than aMSCs [54]. Therefore, even if we do not understand fully why this is the case, human ageing influences greatly the behavior of human MSCs *in vitro*.

1.1.3. Ageing and clinical applications of mesenchymal stem cells

MSCs enter senescence after a finite number of divisions. Hayflick and Moorhead were the first to describe senescence in 1961, demonstrating this "Hayflick limit" for human fibroblasts [55]. MSC proliferation is influenced by donor characteristics and culture conditions, as described in §1.1.2. When senescent on tissue culture polystyrene, MSCs tend to appear relatively large and flat and exhibit prominent actin stress fibers [52] [24]. MSCs from donors of greater age show this type of morphology almost immediately after isolation, and proliferate slowly compared to MSCs from "young" donors [52]. This suggests that an increase in MSC size could be used as a marker of MSC ageing, though this has not been determined conclusively.

In addition, MSCs showed signs of β-galactosidase during long-term culture [56]. This senescence-associated molecule is almost absent at early passages and its presence increases with each passage [51] [56]. β-galactosidase reflects the increase in lysosomal activity seen in senescent cells, and is therefore a marker of MSC ageing, as distinct from ageing of the human donor [57] [58]. In addition, in studies by Roura et al., comparable levels of expression were found in MSCs from both donor age groups (old and young) when analyzed upon fresh isolation from marrow before in vitro expansion [59]. This confirms that increased β-galactosidase presence is a general consequence of *in vitro* culture.

Telomere length reduction is probably the most used marker of cell ageing, by which we mean the number of population doublings (repeated cell cycles and splitting into new cell generations) *in vitro.* Telomeres are DNA repeats (TTAGGG) which protects the end of the chromosome from deterioration or from fusion with neighboring chromosomes. MSCs lose telomeric DNA at a rate of 50 to 100 base pair (bp) per population doubling and reach senescence when telomeres are of critical length [52] [60]. By comparing cells from young and old donors, Baxter *et al.* also estimated using an assay for telomere content in DNA that MSCs were losing 17 bp of telomeric DNA per year *in vivo* [52]. Despite this fact, long telomeres have been observed in senescent MSCs [61]. Therefore, senescence could in fact be controlled by telomere-independent mechanisms. Telomerase* is greatly expressed in cancer cells [62]. Even if Pittenger *et al.* demonstrated telomerase activity in MSCs, other researchers were not able to reproduce those results in MSCs under standard *in vitro*

* An enzyme functions to counteract the loss of telomeric DNA during successive cell divisions by the synthesis of TTAGGG repeats.

culture [63]. Discrepancy in results could be explained by the existence of a few telomerase-positive MSCs. Another study by Simonsen *et al.* claimed that senescence in MSCs could be overcome by forced over-expression of hTERT† [64]. hTERT transduced MSCs proliferated for at least 260 population doublings and maintained osteogenic differentiation capacity both *in vivo* and *in vitro* [65]. However, even if Wang et al. and Rubio et al. confirmed that chromosomal abnormalities were present in early passage cultures [66] [67], those transduced MSCs formed tumor and were altered genetically after injected into immune deficient mice [68]. Despite these results, studies attempting confirmation of these results were not able to show malignant transformation of ten MSC cultures expanded *in vitro* beyond senescence [69]. Wu *et al.* also showed MSCs contained a subpopulation of cells with tumor-initiating capacity [70]. Therefore, putative MSCs that are isolated using today's methods of isolation and identification prior to clinical applications will require robust molecular characterization and karyotype analysis before implantation into patients.

Telomerase also plays another role in MSCs. Its activity correlates with MSC maintainence of expected differentiation potentials *in vitro* [71]. Zhao *et al.* also reported telomerase activity in MSCs was linked in vitro to differentiation [72]. MSCs displayed an increase in telomerase activity during adipogenic differentiation. MSCs from aged donors were shown to have a similar adipogenic and osteogenic differentiation potential at late passage in one study [73] and shown to have a lesser osteogenic differentiation potential [59]. As ageing and senescence of MSCs implies a reduction in differentiation ability, these contradictory results of studies makes it impossible to state a

† hTERT is human Telomerase reverse transcriptase.

clear conclusion regarding lineage fate in long-term expansion. As mechanisms of ageing and senescence of MSCs are largely unknown, senescence is still associated with altered cellular physiology [74].

Recently, senescent MSCs' gene expression profile has been determined and it was concluded that senescence was a continuous process with transcriptional changes detected from the first passage [24]. In addition, this study found an increased expression of five micro RNAs (miRNA‡) in senescent MSCs. The research community does not yet understand how, but miRNA regulates thousands of genes' expression [75]. Therefore, miRNA are currently believed to regulate the onset of senescence.

1.2. Motivations for new studies

MSCs have been heavily studied for the last decades. Accordingly, much research has focused on using MSC properties to treat a range of human diseases. Over 100 clinical trials are ongoing, or will soon be launched, that use MSCs for therapies [76]. In most cases, clinical trials require large numbers of MSCs and therefore need large scale *in vitro* expansion of MSCs. One critical and unresolved issue is that the effects of *in vitro* culture on in *vivo* MSC function are understood poorly. This problem is amplified by the fact that MSC populations show extreme donor-to-donor and intra-population heterogeneity. Population heterogeneity, in addition to differences in MSC isolation protocols and to altered function under large-scale *in vitro* expansion that is highly sensitive to culture protocol details, could explain the discrepancy of results obtained in different laboratories.

‡ miRNA's are short non-coding molecules that are highly conserved and regulate protein expression through interactions with the 3' untranslated region of mRNA

1.2.1. Heterogeneity of adult mesenchymal cells

Research on MSCs over the last ten years has at least led to one conclusion: MSCs are heterogeneous in two ways: among donors and among putative mesenchymal stem cells isolated from a single donor.

Several studies have demonstrated donor-to-donor heterogeneity for MSCs. Phinney *et al.* showed that 17 healthy human donors had disparities in growth rate and osteogenic potential, when cultured and induced toward differentiation *in vitro* under otherwise identical conditions [77]. Other researchers have confirmed these findings and attributed this heterogeneity to different factors such as sampling bias during marrow aspiration [78], age of bone marrow donor [79] and expansion culture protocols [80]. This broad range of findings led researchers to develop isolation method that "homogenize" MSCs. Pittenger *et al.* described an isolation procedure where MSCs were uniformly expressing CD29, CD44, CD71, CD73, CD90, CD105, CD106, CD120a, CD124 and tri-lineage differentiation [3]. Additional surface antigens such as CD271 [81], CD146 [82] and nestin [83] have also been used for the same purpose. However, these studies are not fully conclusive, as antibodies to these markers yielded the entire complement of colony forming unit-fibroblasts from bone marrow – not a subset or subpopulation of those CFU-Fs. We thus conclude that those markers do not uniquely identify MSCs. These studies do not then answer if MSCs express unique surface markers or if those markers represent any value in predicting MSC functions. Several researchers suggested that the answer to this question is no. DiGirolamo *et al.* and Banfi *et al.* showed that MSCs lose their multipotentcy with passaging, without changing in surface-marker phenotype [5] [84]; Maloney et al. have confirmed this result for osteogenic induction in terms of putative MSC surface markers and

alkaline phosphatase expression [85]. Some recent studies showed that plating density greatly influence expression of surface markers such as CD146 [82] and podocalyxin-like protein [86]. Therefore all these studies clearly suggest that MSCs are heterogeneous among donors, and that a combination of surface markers, growth factors and plating density could possibly (but not conclusively) homogenize these populations to select for a "true" stem cell among putative MSCs.

Transcripts expressed by putative MSCs within a given donor source regulate angiogenesis, hematopoiesis, cell motility, and immunity [30] [87]. Based on these findings, single MSCs are unlikely to have all the properties described in the paragraph above. Researchers therefore postulated that subpopulations of MSCs would exhibit some of the properties. Immune staining analysis first supported this hypothesis [87]. Other studies on tri-lineage differentiation potential demonstrated that MSC populations are functionally heterogeneous. For example, Muragila *et al.* analyzed 185 MSCs and showed that they were tripotent, osteochodrongenic or osteogenic progenitors [22]. Muraglia *et al.* did not observed any osteo-adipogenic, adipo-chondrogenic, adipogenic, or chondrogenic MSCs. This suggests that differentiation follows a simple linear progression. Another study working with hTERT-immortalized MSCs identified 7 out of the 8 possible differentiation pathways [88]. More recently, Russell *et al.* claimed that MSCs were differentiating in all 8 possible pathways when cultured under the "right" conditions [89]. They found that about 50% of MSCs were tripotent and less than 5% of MSCs were not differentiating (these percentages are an average from different MSC sources). In these studies, MSCs were mostly exhibiting tri, osteo-chondrogenic and osteogenic potentials. This was consistent with studies performed by Pittenger *et al.* and Banfi *et al.* [3] [84]. Subsequent studies by Russell *et al.* showed that tripotent MSCs

proliferated faster and had a lower rate of apoptosis than unipotent MSCs [90]. Together these studies suggest that multi ineage differentiation potential is hierarchically structured in MSCs. Since in those studies only 50% of MSCs expanded and 50% of those "expanded" cells were tripotent, 1 out of 4 putative MSCs can be considered tripotent. Based on these data, it appears that tripotent MSCs then proliferate to produce more bipotent cells, and bipotent MSCs yield unipotent MSCs with higher rate of apoptosis. It is unclear at present whether uni- or bipotent MSCs can reverse or change their commitment, and we do not yet know how and why MSCs undergo those restricted differentiation pathways. However, Phinney et al. claimed that WNT and BMP signaling pathways including DKK1, DKK3, and Gremlin are more abundant in "daughter" MSCs as compared to "parent" MSCs, and that these signaling pathway markers are enriched in tripotent cells. These data indicate that MSCs use paracrine or autocrine mechanisms to select their lineage specification. Together, all of these studies clearly show that putative MSC populations that are isolated and expanded *in vitro* are a heterogeneous cell population, and that lineage specification is a very complex process.

1.2.2. Potential clinical applications of fetal mesenchymal stem cells

As mentioned in §1.1.1, aMSCs are isolated from bone marrow and expanded *in vitro* while retaining a degree of multipotency [3]. Therefore, they are of interest for stem cell-based therapy [91] [92]. However, aMSCs' low prevalence in bone marrow, high degree of senescence with increased proliferation, limited proliferation capacity [21] [93], and limited differentiation potential [94] [89] have restricted full realization of their clinical applications. Alternative

MSCs from fetal sources (fMSCs) have been isolated more recently [95] [96] [97] [98]. Although the basic biology, immunogenicity and differentiation potential of fMSCs are not fully explored and understood at present, fMSCs have been reported to exhibit superior osteogenic and myogenic capacities both *in vitro* and *in vivo*, as compared to MSCs from adult sources (aMSCs) [99] [96]. Zhang *et al.* also showed that fMSCs have greater proliferation capacities *in vitro* than aMSCs. Min *et al.* also demonstrated that fMSCs co-transplantation with aMSCs improved heart function, compared to injection of only aMSCs [100]. These studies lead some researchers to consider fMSCs as offering a more promising candidate for clinical applications. However, and as mentioned later in § 3.1, fMSCs also face some serious practical and ethical issues for stem cell-based clinical trials and therapy.

1.2.3. Heterogeneity of mesenchymal stem cells influences clinical applications

MSCs are currently under investigation at various stages of clinical trials to treat several diseases such as heart attack [101], spinal-cord injuries [102], muscular dystrophy [103], bone fractures [104], etc. Currently, there is no standardized manufacturing process, but most companies that provide cells for such clinical applications follow 'good manufacturing practice' and measure sterility, viability and chromosomal stability to meet FDA requirements. Surprisingly, Phase I/II trials that have an unrestricted number of donors use minimally expanded MSCs, to reduce the possibility of altering cellular composition and function. However, if the desired function is only expressed in a minor subpopulation of the delivered cells, minimal expansion could result in poor potency in applications. Contrastingly,

when the number of donors is restricted or when the trials require very large number of cells, MSCs are produced in "batches". This "batch"-like expansion introduces bias into culture conditions that can influence MSC functionality and clinical endpoints and efficacy. While *in vivo* selection is influenced by several unknown factors including cell-based mechanisms or extrinsic chemicals, *in vitro* expansion affects intra-population heterogeneity in unexplored ways. In addition, the effects are rarely evaluated post-expansion, and it is therefore possible that large-scale expansion selects or rejects a particular subpopulation. This possible narrowing of the population could thereby enhance or reduce efficiency of MSCs for treatment.

Moreover, Seeger *et al.* showed that MSCs expanded with the same protocol at different time and expanded with different protocols were different [105]. These results, coupled with the number of studies showing that MSCs are functionally heterogeneous, implies that researchers must develop and perform experiments that predict or qualify MSC potency before using those cells for clinical applications. For example, Kuznetsov *et al.* showed that *in vitro* osteogenic differentiation assays do not reveal the ability of MSCs to form osseous tissue *in vivo* [36]. Another study using MSCs to treat aGVHD[§] demonstrated that there was no correlation between *in vitro* suppression by MSCs in mixed lymphocyte cultures and immunologic effects *in vivo* and long-term survival [106]. Therefore, donor-to-donor and intra-population heterogeneity and effects of long-term expansion on MSCs could explain discrepancy in results on stem cell-based therapy. In general, we see a poor correspondence between *in*

[§] aGVHD (acute graft-versus-host disease) is a common complication following an allogeneic tissue transplant. Immune cells (white blood cells) in the tissue (the graft) recognize the recipient (the host) as "foreign". The transplanted immune cells then attack the host's body cells.

vitro demonstration of successful cell therapies and *in vivo* realization [107] [108].

1.3.Aims

aMSCs are potentially highly effective for clinical applications and lack the ethical issues that fMSCs have faced. However, aMSCs have been shown heterogeneous (as discussed above) and difficult to characterize at a single cell level. These conditions lead to the question: Are there true mesenchymal stem cells in the stromal cells derived from bone marrow, or just a mixed population of adipogenic, chondrogenic, and osteogenic precursor cells? This question is important to understand MSC biology, and could be crucial for some therapeutic settings.

Phinney conjectured that aMSCs derive from fMSCs [109]. In addition, fMSCs display superior proliferation, differentiation capacities, homogeneous population [17] and have been considered by some to be ideal for tissue-regenerative therapy. We therefore hypothesized in this thesis that putative aMSC populations are heterogeneous, but contain "true" stem cells with respect to self-renewal proliferation capacity and multipotent (at least tripotent) differentiation potential.

In this thesis, this hypothesis was tested in terms of three characteristics of MSCs, and is reported in the four following chapters:

Chapters 2: First, as culture conditions greatly influence proliferation and differentiation potentials of MSCs *in vitro*, we sought to quantify whether and how the different conditions used in our laboratory yielded different proliferation rates or cell morphologies in the attached state.

Chapter 3: Using standardized *in vitro* culture conditions, we then considered identification of an aMSC subpopulation that exhibited a "size" or diameter similar to that of the comparably homogeneous fMSCs. We considered cell diameter first, as Sekiya *et al.* showed that cell size and morphology greatly influence proliferation and differentiation potentials [45], and as fMSCs have been shown smaller and homogeneous in size than aMSCs in the attached state [96]. The method used for this diameter-based identification and isolation of cell subpopulations was achieved in collaboration with the Han group (MIT and SMART BioSyM) who designed and fabricated a device capable of relatively high-throughput sorting of cells on the basis of suspended cell diameter [110].

Chapter 4: Then, as researchers have shown that *in vitro* migration is correlated to *in vivo* migration (via the cell surface marker CXCR4), and as fMSCs have been shown to migrate faster than most other cell types [96], we studied the migration speed of those "sorted" subpopulations as compared to fMSC migration speed.

Chapter 5: Finally, we compared the mechanical properties of aMSC subpopulations to those of fMSCs, in terms of transit time through a mechanical constriction under fluid flow.

Chapter 6: We close with general conclusions and resolution of our initial hypothesis – that these characteristics of size, migration velocity, and mechanical properties may identify a subpopulation within putative aMSCs that is well matched in those characteristics to fMSCs – and discuss the implications for future biological and engineering studies as well as clinical applications.

Chapter 2. Media, plastics, and culturing protocols

2.1.Study background, hypothesis and design

Human mesenchymal stromal or stem cells (MSCs) are multipotent cells capable of differentiating *in vitro* along multiple cell lineage pathways such as osteoblasts, myoblasts, and neurons [3] [111] [112] [113]. MSCs are believed to leave their *in vivo* tissue niche [114], and to differentiate within a range of microenvironments as diverse as brain, muscle, and bone [115] [116] [117]. This multilineage ability coupled with MSCs' relative easy isolation from bone marrow and *in vitro* expansion capacity have prompted researchers to explore use of MSCs as therapy for a variety of diseases [118]. Clinical trials involving MSCs have been reported for treatment of osteogenesis imperfecta [119], metabolic diseases [120], amyotrophic lateral sclerosis [121] and myocardial infarction [122], with mixed results. Safety, feasibility and efficacy of MSCs as therapeutic agents are currently under investigation. Several studies using large numbers of MSCs per experiment, up to 10^9 [122], have reported inconsistent results regarding MSCs proliferation, morphology, and differentiation abilities. This discrepancy could be explained by the lack of standardization in isolation and expansion protocols among laboratories, which led to large heterogeneity among MSCs in both *in vitro* experiments and clinical trials. The lack of homogeneity in MSC populations has become problematic for MSC research, as results cannot be compared from one laboratory to another. Commercial sources of adult MSCs derived from human bone marrow, such as Lonza Walkersville (Gaithersburg, MD), Invitrogen (Carlsbad, CA), and Stem Cell Technologies (Vancouver, BC, Canada), have attempted to produce standard cell phenotypes during MSC expansion via culture protocols and media content. Despite those companies' efforts, MSCs remain heterogeneous both within a

population from a given bone marrow source [45] and among populations derived from different human bone marrow sources.

In addition to the heterogeneity of MSC populations, the lack of standardization in culture conditions – especially regarding initial seeding and passaging densities [123] [45] [124], the serum selection [125], the culture surfaces [126], and the effect of donor age and cryopreservation [127] – has obviated clear comparison among results obtained in different labs and clinics. Several teams have published in the literature work on factors modifying MSC proliferation and morphology. For example, Shahdafar *et al.* [128] studied MSC expansion using different media and concluded that autologous serum gave the highest proliferation rate. A short overview of the key works published is summarized in Table 1. Colter *et al.* even showed that in change in proliferation rate correlates with a change in morphology [17]. We see from these studies that several factors influence MSC proliferation, morphology and the capacity and extent of differentiation *in vitro*.

Our laboratory uses a few different types of culture dishes, media and culture protocols. Therefore, the first study of this thesis focuses on understanding how those different conditions could influence the measurable characteristics of MSCs *in vitro*. This study aims to identify "our best condition" for our MSCs to grow. We thus seeded MSCs at 3 densities onto 3 different dishes using two media. I summarized all conditions in Table 2. The point of this study is not to determine the "ideal condition" for MSC *in vitro* culture expansion in general, but to determine the "best condition" to maximize cell proliferation rates (growth rates) and obtain uniform morphology for MSCs cultured inside our laboratory considering the variation in tissue culture products and protocols that we use.

Table 1: Reports of MSC growth rate under variations of culture conditions.

Group	Notes
Shahdadfar et al., 2005 [128]	Compared hMSCs morphology and growth with "Autologous Serum" and "Fetal Bovine Serum" medium.
Moroni et al., 2006 [129]	Studied the effect of different scaffolds and of their surface nanotopology on cell seeding, attachment, and proliferation.
Serakinci et al., 2004 [68]	Used adult mesenchymal stem cells (hMSC) transduced with the telomerase hTERT gene to investigate the neoplastic potential of adult stem cells.
Riddle et al., 2006 [130]	Studied the influence of mechanically induced fluid flow on human mesenchymal stem cell proliferation.
Peister et al., 2004 [131]	Developed a protocol that provides rapidly expanding MSCs.
Sotiropoulou et al., 2006 [44]	Attempted to identify the optimal protocol for the large-scale production of MSCs by comparing various culture conditions.
Castelas et al., 2006 [132]	Analyzed hMSC morphology and proliferation after up to 28 days of incubation in eight different formulations of fibrin gels.
Doucet et al., 2005 [133]	Studied influence of PL-containing medium enriched by growth factors (platelet-derived growth factors (PDGFs), basic fibroblast growth factor (bFGF), transforming growth factor (TGF-b), insulin-like growth factor-1 (IGF-1) on hMSC expansion.
Banfi et al., 2000 [84]	Investigated the effects of *in vitro* expansion on MSC pluripotentiality, proliferative ability, and bone-forming efficiency in vivo.

2.2.Materials and Methods

2.2.1. Establishment of hMSC culture

Adult mesenchymal stem cells (referred later as aMSCs) were purchased from commercial sources: ReachBio human bone marrow mesenchymal stem cells – lot number 0090408 (referred later as RB1 – ReachBio LLC, Seattle, WA), and Poietics™ human mesenchymal stem cells – lot number 7F3675 (referred later as PL2 – Lonza Walkersville Inc., Gaithersburg, MD). All aMSCs were cultured in MesenCult® media and maintained at 37°C in a humidified atmosphere containing 5% CO_2. Medium was changed every 3 days, and cells were passaged when reaching 80% confluence (see Appendix A for passaging protocol).

2.2.2. Preparation and characterization of media

Two liquied culture media sources were used for aMSCs: MesenCult® medium and our homemade medium. MesenCult® media was made of 90% MesenCult® basal media plus 10% MesenCult® supplements of proprietary composition, purchased from StemCell Technologies (Vancouver, BC, Canada). Our homemade medium is constituted by 90% Gibco® low-glucose Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) plus 10% selected lot fetal bovine serum or FBS (Lot # 696409, Invitrogen, Carlsbad, CA). Both types of media were mixed at room temperature and filtered for sterility using Nalgene MF75™ Series filter (Product # 295-3345 - Thermo Fisher Scientific, Rochester, NY).

2.2.3. Preparation and characterization of plastics

Cell behavior was investigated on three surfaces: BD Falcon™ 175 cm^2 Cell Culture Flask comprising polystyrene (Product # 353028 - BD, Franklin Lakes, NJ), Nunc EasYFlasks™ Nunclon™ Δ *175* cm^2 flask comprising polystyrene (Product # 159910 – Nalge Nunc International, Rochester, NY), and fibronectin coated 35 mm glass-bottom dish with 20 mm micro-well #0 cover glass (Product # D35-20-0-N – In Vitro Scientific, Sunnyvale, CA). These dishes will be referred later as Falcon T175, Nunc T175, and Petri Dish respectively for BD Falcon™ 175 cm^2 Cell Culture Flask, Nunc EasYFlasks™ Nunclon™ Δ *175* cm^2 flask and fibronectin coated 35 mm glass bottom dishes glass bottom dish with 20 mm micro-well #0 cover glass.
The glass bottom dishes were functionalized for cell proliferation by adding a fibronectin** solution (1 mg/mL – 1 in phosphate buffered saline – SIGMA F2006 fibronectin from human plasma, Sigma-Aldrich, St. Louis, MO) following the protocol described at http://www.invitrosci.com/.

2.2.4. Cell Culture, observation and analysis

aMSCs at passage 6 (i.e., after six repeated cycles of attachment-expansion-trypsinization-reseeding at lower density of cells/cm^2 as describe in Appendix A) were plated in triplicate at 100, 500 and 2000 cells/cm^2 onto the different dishes. The media was changed every 3 days for a total duration of 12 days. The cells were detached with trypsin EDTA†† 1X (0.05% trypsin/0.53mM EDTA in HBSS without sodium bicarbonate calcium and magnesium – MediaTech Inc., Manassas VA – referred to hereafter as T/E), and the cells from each

** Fibronectin is a high-molecular weight glycoprotein of the extracellular matrix that binds to membrane-spanning receptor proteins called integrins
†† EDTA stands for Ethylenediaminetetraacetic acid

plate were counted in duplicate with a hemocytometer. Morphology of MSCs was not quantified and was just noticed by eye looking at the MSCs with a microscope. This is why we will not draw any conclusion on the influence of culture conditions on morphology, though we note that no evident changes were observed from one condition to another.

The purpose of these experiments was to determine if the different culture conditions we used in our laboratory would influence the MSCs' proliferation rate and morphology. The experiments were designed to simply quantify both proliferation rate and morphology of the cells. The experiments done are summarized in Table 2.

Table 2: Summary of the experiments performed to complete Chapter 2 study

	Homemade Media	MesenCult® Media	Falcon T175
Experiment 1: RB1 cells	MSCs seeded at 3 different density: 100, 500 and 2000 cells/cm^2	MSCs seeded at 3 different density: 100, 500 and 2000 cells/cm^2	
	MSCs seeded at 3 different density: 100, 500 and 2000 cells/cm^2	MSCs seeded at 3 different density: 100, 500 and 2000 cells/cm^2	**Nunc T175**
	MSCs seeded at 3 different density: 100, 500 and 2000 cells/cm^2	MSCs seeded at 3 different density: 100, 500 and 2000 cells/cm^2	**Petri dish**
Experiment 2: PL2 cells	MSCs seeded at 3 different density: 100, 500 and 2000 cells/cm^2	MSCs seeded at 3 different density: 100, 500 and 2000 cells/cm^2	**Falcon T175**
	MSCs seeded at 3 different density: 100, 500 and 2000 cells/cm^2	MSCs seeded at 3 different density: 100, 500 and 2000 cells/cm^2	**Nunc T175**
	MSCs seeded at 3 different density: 100, 500 and 2000 cells/cm^2	MSCs seeded at 3 different density: 100, 500 and 2000 cells/cm^2	**Petri dish**

2.3.Results

2.3.1. Media and culture surfaces considered do not influence MSC growth and morphology

The experiments were performed in triplicate for the 2 MSC sources mentioned above. For each source, we plated the cells at passage 6 and cultured them for 12 days to quantify any change in MSC proliferation rate (in terms of the number of population doublings/day). As observed previously with human and rat MSCs [5] [134], the cultures showed a lag period with little expansion in the first 3 days. Thereafter the cell population expanded rapidly. The rates of expansion of the cultures were not sensitive to the media and tissue culture surfaces/vessels used for both MSC sources. These data are summarized in Figure 1.

In this work, we assume that all cells adhered; even it is rarely the case. This could lead to an underestimation of the average cell proliferation. However, this underestimation does not change our conclusion on the influence of media and culture surfaces on cell growth. Indeed, we assume that, in average, the number of cells not attaching is about the same under those different conditions.

Even if the rates of expansion were insensitive to the media and plastics used, the rates of expansion for the two different MSC sources were very different. This represents the heterogeneity of MSC populations discussed in § 4.1.

We conclude from this study that media and culture surfaces/vessels used in our laboratory do not influence MSC proliferation rates, or at

least these factors do not influence MSC proliferation rates differently for a given cell source.

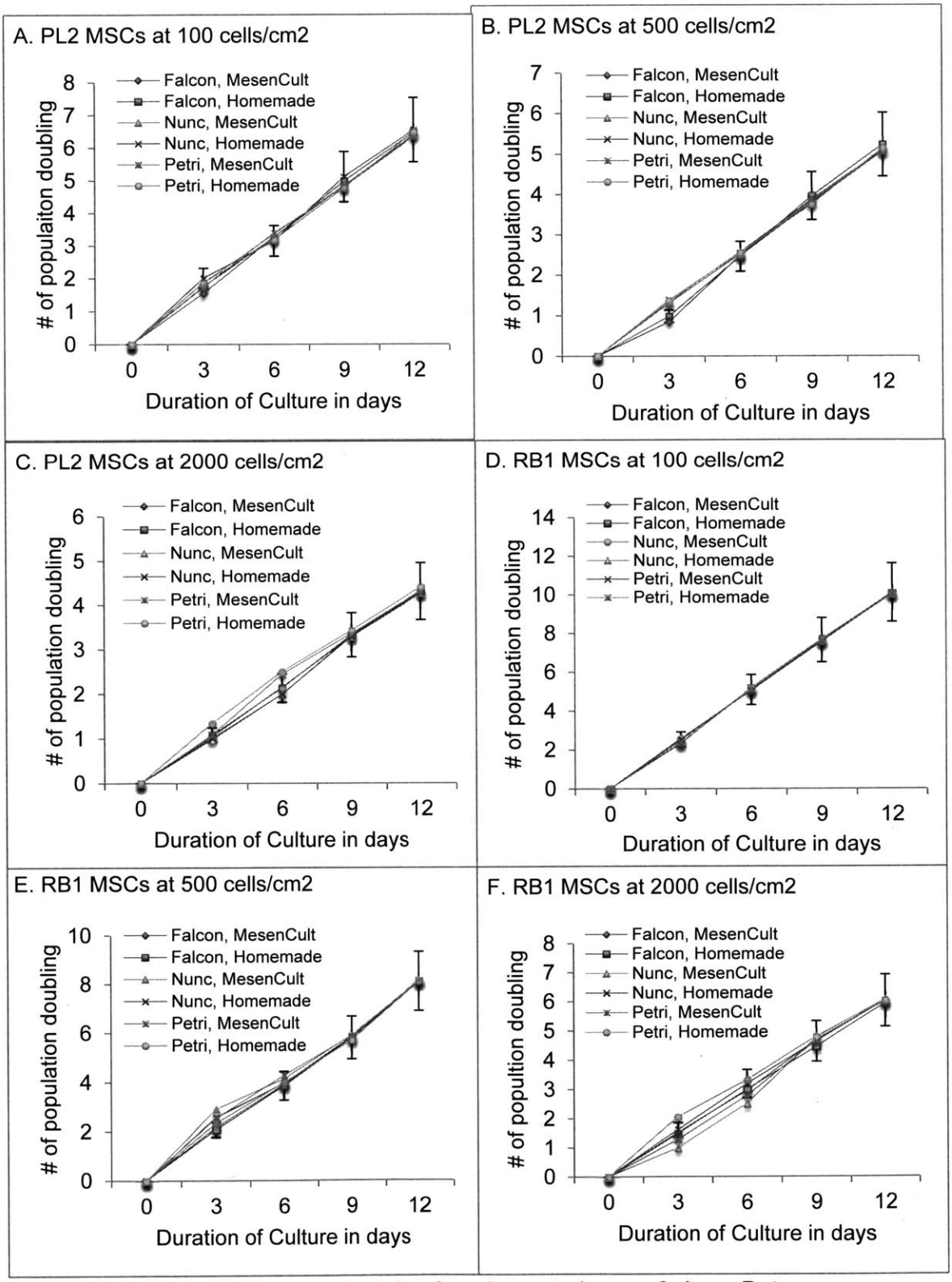
In addition, Figure 1 can be deceiving: some graphs do not have a high enough resolution, proliferation rates were never exactly equal to one another even if they seem so. Proliferation were definitely close, but as we conclude form the study that proliferation rates did not depend on medium and plastics in our laboratory, it was not interesting to enlarge the scale to show all the details. Exact results are summarized in Table 3.

Table 3: Number of RB1 and PL2 aMSCs population doubling under different culture conditions

RB1						
Dish	**Falcon**					
Density	100		500		2000	
Medium	*Mesen Cult*	*Home made*	*Mesen Cult*	*Home made*	*Mesen Cult*	*Home made*
Days of culture	# pop doubl	# pop doubl	# pop doubl	# pop doubl	# pop doubl	# pop doubl
0	0	0	0	0	0	0
3	2.54	2.32	2.17	2.09	1.51	1.48
6	5.09	5.19	3.91	3.86	2.94	2.97
9	7.64	7.68	5.85	5.81	4.67	4.43
12	10.10	10.07	8.10	8.11	6.01	5.93
Dish	**Nunc**					
Density	100		500		2000	
Medium	*Mesen Cult*	*Home made*	*Mesen Cult*	*Home made*	*Mesen Cult*	*Home made*
Days of culture	# pop doubl	# pop doubl	# pop doubl	# pop doubl	# pop doubl	# pop doubl
0	0	0	0	0	0	0
3	2.59	2.46	2.91	2.58	0.99	1.61
6	5.07	5.16	4.14	3.96	2.50	3.17
9	7.58	7.61	5.92	5.84	4.74	4.62
12	10.01	10.04	8.13	8.07	6.08	6.02
Dish	**Petri**					
Density	100		500		2000	
Medium	*Mesen Cult*	*Home made*	*Mesen Cult*	*Home made*	*Mesen Cult*	*Home made*
Days of culture	# pop doubl	# pop doubl	# pop doubl	# pop doubl	# pop doubl	# pop doubl
0	0	0	0	0	0	0
3	2.53	2.36	2.55	2.34	1.27	2.04
6	5.04	5.11	4.29	3.99	2.76	3.30
9	7.56	7.61	5.88	5.71	4.64	4.78
12	10.04	10.04	8.16	8.12	6.03	6.03

PL2						
Dish	**Falcon**					
Density	100		500		2000	
Medium	*Mesen Cult*	*Home made*	*Mesen Cult*	*Home made*	*Mesen Cult*	*Home made*
Days of culture	# pop doubl	# pop doubl	# pop doubl	# pop doubl	# pop doubl	# pop doubl
0	0	0	0	0	0	0
3	1.55	1.75	0.84	0.98	1.05	1.08
6	3.27	3.26	2.48	2.46	2.15	2.13
9	4.84	4.98	3.80	3.97	3.29	3.32
12	6.38	6.48	5.06	5.23	4.26	4.30
Dish	**Nunc**					
Density	100		500		2000	
Medium	*Mesen Cult*	*Home made*	*Mesen Cult*	*Home made*	*Mesen Cult*	*Home made*
Days of culture	# pop doubl	# pop doubl	# pop doubl	# pop doubl	# pop doubl	# pop doubl
0	0	0	0	0	0	0
3	1.88	2.01	1.33	1.38	0.98	1.01
6	3.36	3.15	2.58	2.55	2.00	1.99
9	4.85	5.11	3.86	3.89	3.28	3.29
12	6.40	6.55	5.08	5.09	4.27	4.29
Dish	**Petri**					
Density	100		500		2000	
Medium	*Mesen Cult*	*Home made*	*Mesen Cult*	*Home made*	*Mesen Cult*	*Home made*
Days of culture	# pop doubl	# pop doubl	# pop doubl	# pop doubl	# pop doubl	# pop doubl
0	0	0	0	0	0	0
3	1.73	1.86	1.30	1.36	1.09	1.34
6	3.40	3.18	2.51	2.54	2.44	2.50
9	4.81	4.78	3.84	3.76	3.35	3.42
12	6.35	6.48	5.06	5.15	4.32	4.41

Figure 1: Number of population doubling as a function of media and dishes.



MSCs were plated at passage 6 at day 0 and counted every 3 days. Data are expressed as the mean ± SE. On this Figure, "MesenCult" stands for MSCs cultured with MesenCult medium; "Falcon" stands for MSCs cultured with Falcon T175 dishes, etc.

2.3.2. Culturing protocols change cell behavior drastically

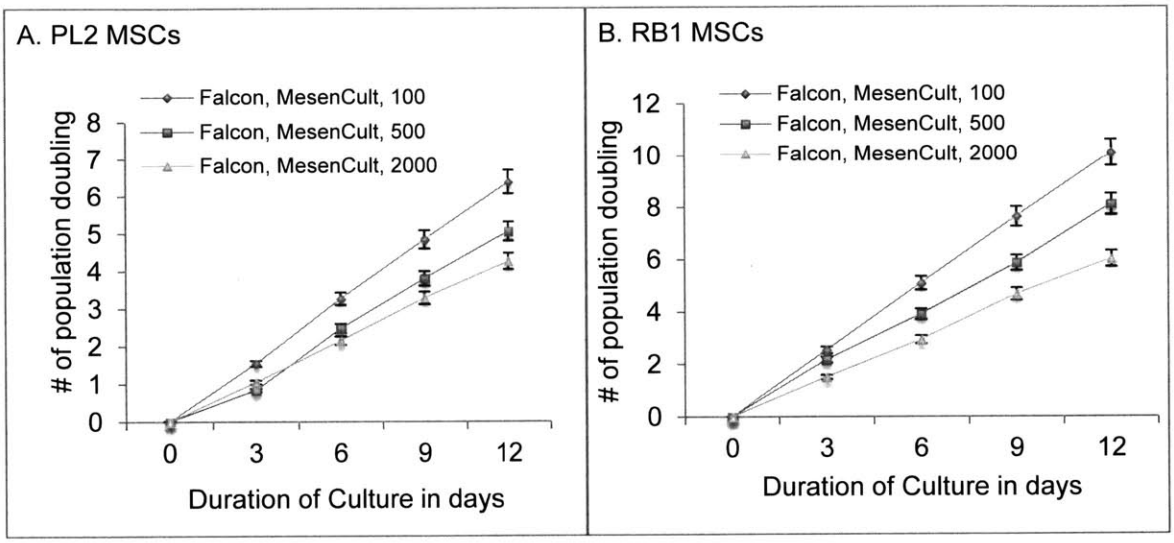
Here we plated the "passage 6" cells at different densities in triplicate and cultured them for 12 days to quantify any change in MSC proliferation rate. The point was to study the effect of initial cell seeding density on MSC proliferation rate. As we showed in the previous paragraph that medium and plastics are interchangeable regarding MSC proliferation, we therefore did not graph all data for clarity and draw conclusion for all media and dishes used in our laboratory. The cultures showed a lag period with little expansion in the first 3 days. We found that the rates of expansion of the cultures were extremely sensitive to the initial seeding densities for both MSC sources. These data are summarized in Figure 2.

We conclude form this work that MSCs proliferation is greatly dependent on initial seeding density. MSCs proliferation was inversely proportional to seeding density. Therefore, RB1 MSCs plated at 100 cells/cm^2 had 10 population doublings whereas RB1 MSCs plated at 2000 cells/cm^2 had only 6 population doublings. We can draw the same conclusions for PL2; PL2 MSCs plated at 100 cells/cm^2 had more than 6 population doublings whereas PL2 MSCs plated at 2000 cells/cm^2 had only 4 population doublings. The impressive difference could have come from the fact that MSCs seeded at 2000 cells/cm^2 became senescent due to contact with other cells and therefore stopped dividing. However, throughout the experiment, MSCs did not reach 80% confluence (checked visually), which indicates that MSCs did not enter senescence by contact inhibition. We therefore concluded that high density seeding correlated with the MSCs to divide slower. We observed once again strong heterogeneity between MSC populations from two different patient sources. We can also

make the same comment as in the previous section about some deceiving graphs in Figure 2.

Figure 2: Effect of initial seeding densities on aMSC proliferation rate.

aMSCs were plated at passage 6 at day 0. Data are expressed as the mean ± SE. On this Figure, "MesenCult" stands for MSCs cultured with MesenCult medium; "Falcon" stands for MSCs cultured with Falcon T175 dishes. Cultures were counted every 3 days.



(A) Expansion as a function of initial seeding densities for PL2. (B) Expansion as a function of initial seeding densities for RB1.

2.4.Outcome

2.4.1. Conclusions

We conclude from this study that media and dishes used in our laboratory do not influence MSC proliferation, or at least influence proliferation in the same terms. However, we see from figure 2 that initial cell seeding density seems to influence MSC proliferation rate.

These conclusions are qualified statements, as untested materials could lead to other findings and differences. However, the test of three different types of dishes strongly suggests that culture surfaces with "high" young modulus (greater than 2GPa) [135] do not influence MSC proliferation. In addition, in contrast with our findings, some researchers have reported a MSC proliferation modification in function of medium used [128]. This discrepancy could be due to several factors. MesenCult® medium contains 10% supplements, which could very well be some sort of FBS. Therefore both media we used could be similar enough to not induce a difference in MSC proliferation. This is one of possible explanation amongst many others. Concerning our conclusions on seeding density dependence of MSC proliferation rate, most reports in the literature agree with our findings [134] [136]. The reason why seeding density affects proliferation rate is still unclear, and is under still investigation. One of the possible explanations could be that biological, chemical, mechanical cues or a combination of these lead MSCs to enter senescence and stop proliferating, and thus regulate their proliferation. This hypothesis could actually be interesting to test and is a possibility for continued research.

This study enables us to use both the above media and plastics interchangeably during our studies in our laboratory. Any other practical applications of this work still require careful consideration, as we have drawn conclusions only for products used in our laboratory. The present study did not aim to resolve the general question on the influence of media and/or dishes and/or seeding densities over cell growth and morphology, and we cannot extend our conclusions outside of the delimitations described above based on available experimental data.

2.4.2. Possibilities for continued research

There are many appealing directions available to continue this research. Three are described here. First, and as mentioned above, one could study if biological, chemical, mechanical cues or a combination of these lead MSCs to enter senescence and stop proliferating. This could be studied by seeding MSCs at different densities, and measuring proliferation rates over longer period of times that considered here. Second, one property of culture vessel surfaces that one could study is nanotopography, which could modify MSCs response and lead to a change in proliferation or morphology. At last, one could also study if culture conditions influence the entire entire population or just a subpopulation. This study could give insights into the heterogeneity of MSCs, and could also provide information on possible culture conditions that would "select" homogeneous subpopulation of MSCs.

Chapter 3. Cell size distribution

3.1.Study background, hypothesis and design

Fetal mesenchymal stem cells (referred to hereafter as fMSCs) are multipotent and can differentiate *in vitro* along the osteogenic, adipogenic and chondrogenic lineages [137]. These cells have long been considered to treat degenerative diseases and injuries. Indeed, fMSCs derived from fetal bone marrow are more plastic‡‡ and grow faster than aMSCs [53], and have potential for allogenic transplantation [138]. The concept of such cell-based therapy lies in the generation of healthy cells that replace or augment behavior of the lost or damaged ones, respectively, though the *in vivo* details remain under debate. For example, this treatment has been posited as useful in the dysfunction of contractile heart muscle after a heart attack [101], or in the pancreas where new β-cells could secrete insulin [139]. Moreover, implanting stem cells into the spinal cord did allow new nerve cells to restore motor function in people with spinal-cord injuries [102]. Muscular dystrophy [103] and bone fractures [104] could also be treated using fMSCs as they show superior osteogenic and myogenic capacities thanperinatnal and adult MSCs [99] [96]. However, fMSC research has encountered several issues, including the risk of teratoma formation [140] [141] – a benign tumor caused by the presence of contaminating undifferentiated cells if co-transplanted with differentiated cells – or the risk of immune response. In addition to these issues, fMSC research raises ethical debates in many countries, as the use of fMSCs as a therapeutic agent implies the destruction of the fetus [142]. All these issues led researchers to look for alternative approaches and cell sources. One of the most promising approaches is the use of adult mesenchymal stem cells (referred to hereafter as aMSCs). These

‡‡ plastic means capacity to differentiate into one or more lineages

cells are easier to obtain [140] [141]. aMSCs could be implanted without intentional chemical differentiation or extended culture to increase cell number if derived from a stem cell rich source such as bone marrow [143]. aMSCs could also be easily used autologously[§§], and have therefore led to rapid clinical application [144]. Surgeons have already proved the concept by taking some bone marrow, and re-implanting them into the patient to replace some lost or damaged cells [145] [146] [101]. These advantages of aMSCs compared to fMSCs could explain why the National Institutes of Health invested USD 4,634 billion on stem-cell research in the period 2007-2011, and only 11 % of this amount was spent on human fetal stem cell research (http://report.nih.gov/categorical_spending.aspx).

However, and despite all these advantages, aMSCs are actually not a single cell type as currently isolated and defined, but rather a heterogeneous population of cells that exhibits a range of differentiation potential [147] [143] and exhibits poor overall integration and survival in host tissues [148]. Therefore, an interesting hypothesis for stem cell-based therapy would be to find a subpopulatin of aMSCs that share certain measureable properties with the clinically useful, comparably homogeneous population of fMSCs. Such an approach would, if successful, combine the clinical advantages of fMSCs with the practical and ethical advantages of aMSCs. Thus, the second work in this thesis focuses on one of the simpler physical characteristic to study: the diameter of the cell.

As one indicator of this cell population heterogeneity, aMSCs exhibit a broader diameter distribution than do fMSCs, ranging in diameter from 10 to 30 μm [17]. As this work aims to isolate a subpopulation of aMSCs similar

[§§] autologously means derived and transplanted from the same patient

in diameter to fMSCs, we needed to separate aMSC by diameter. We chose to use a microfluidics inertial migration based device to achieve this goal. Spiral microfluidics-based cell separation systems offers great advantages over conventional cell sorting techniques such as FACS. These advantages include reduced sample volume; faster sample processing that reduces analysis time; and high sensitivity and spatial resolution [149] [150]. Several other microfluidics separation techniques have also been explored recently, such as dielectrophoresis [151], free flow acoustophoresis [152], hydrophoresis [153] and hydrodynamic filtration [154]. Even if these other technologies are useful tools for diameter-based cell separation, these devices are costly, and require an external force field for functionality that could possibly affect cell viability. In addition, all of these other methods have lower throughput than current spiral microfluidics approaches, which limits adoption of those methods in the biological engineering or clinical communities [149]. Hence, the clear advantage for the present study is to use the high throughput, simple, and relatively cheap inertial migration technique for diameter-based cell separation.

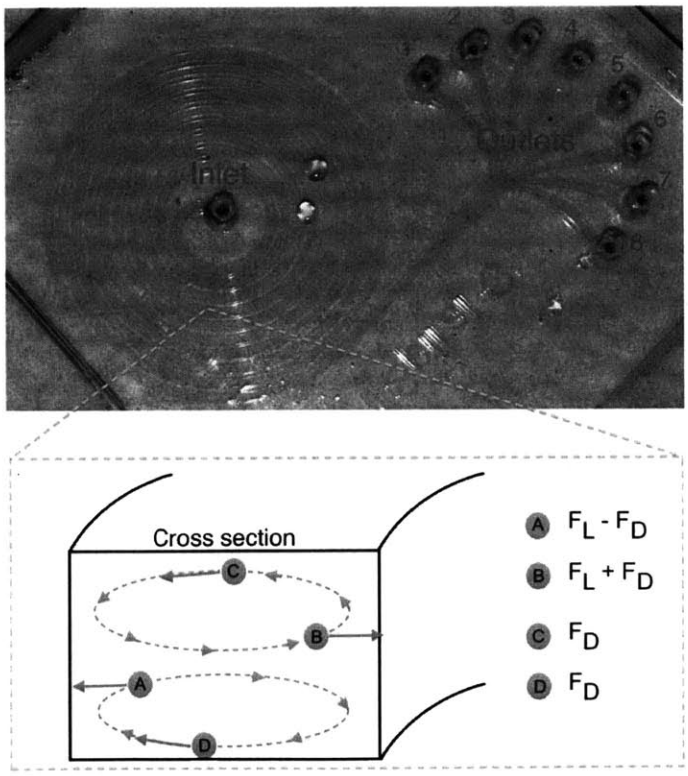
3.2.Materials and Methods

3.2.1. Design and characterization of the 'separation' microfluidic device

Recently, diameter-based cell separation in microfluidic systems has been demonstrated based on principles of inertial migration [155] [156]. As the technique is membrane-free, it runs in a continuous flow that allow high throughput and does not require an external force to work. Seo et al. demonstrated this principle of centrifugal separation [156]. Here we use a passive spiral microfluidic device (Figure 3) that was designed by Dr. Ali Bhagat (SMART BioSyM), improved in its

operation protocol by Mr. Jacky Lee (Ph.D. candidate, National University of Singapore), and fabricated by Ms. Sha Huang (Ph.D. candidate, MIT), all working under the supervision of Prof. Jay Han (MIT EECS and BE). This device employs centrifugal-based differential migration to separate cells by size.

Figure 3: Passive spiral microfluidic device for separation of particles using centrifugal-based differential migration and schematic of the forces experienced by the particles, which results in differential migration within the microchannel.



Under Poiseuille flow conditions in a spiral channel with rectangular cross-section, the inertial migration lies on the balance of dean drag

forces and lift forces schematically illustrated in Figure 3. Ookarawa *et al.* and Asmolov *et al.* derived respectively the Dean drag forces [157] and the lift forces [158]:

Dean drag force: $F_D = 5.4x10^{-4}.\pi.\mu.De^{1.63}.a_p$ (N) [157]

Lift force: $F_L = \rho.G^2.C_L.a_p^4$ (N) [158]

where Dean number (*De*): $De = Re.\sqrt{(D_h/2R)}$ [159],
R: radius of curvature (m) of the path of the channel,
Re: the flow Reynolds number, D_h: micro channel hydraulic diameter (m), μ: fluid viscosity ($kg.m^{-1}.s^{-1}$), a_p: particle diameter, ρ: density of fluid medium ($kg.m^{-3}$), *G*: shear rate of the fluid (s^{-1}) $G= U_{max}/D_h$,
C_L: lift coefficient, U_{max}: maximum fluid velocity ($m.s^{-1}$)

The centrifugal acceleration leads to the formation of two counter-rotating vortices known as Dean vortices in the top and bottom halves of the channel [160] [161]. These forces equilibrate the particles at a distinct position across the channel section [162]. However, Di Carlo *et al.* showed that the particles tend to occupy a single equilibrium position in a spiral channel only if $a_p/D_h > 0.07$ (where a_p is the particle diameter and D_h is the channel hydraulic diameter) [163] [164]. In addition to these "cross-sectional considerations," the particles (or cells) are not focusing on one position instantly. Therefore, we need to consider the minimal channel length necessary for the particles to migrate. Ookawara *et al.* derived this necessary channel length (L_l) for the particles to migrate, using Asomolov's lift force equation and assuming Stokes drag [157]:

$$L_I = \frac{U_f}{U_L} \times L_M \text{ (m)}$$

where U_f is the average fluid velocity (m.s^{-1}), U_L the particle lateral migration velocity (m.s^{-1}) ***, and L_M the migration length (m)

Given those considerations and the diameter range of the hMSCs, the present device was designed with a cross section of 200µm height and 500µm wide.

Before using the device for separating hMSCs, we prepared it following the protocol describe in Appendix B. When the device was readied, we injected the hMSCs into the device using a syringe pump at a constant rate of 1.5 mL/min. We then collected the cells at the outlets and analyzed then using the protocol described later in §3.2.3. To later identify the sorted aMSCs, we will name them "Outlet i" where i is the number representing the channel form which the aMSCs were collected (see Figure 3 channel's number).

*** The lateral migration velocity is given by Bhagat et al. [159] as $U_L = \frac{\rho U_{max}^2 a_p^3 C_L}{3\pi\mu D_h^2}$

3.2.2. Establishment of hMSC culture

3.2.2.1. Adult human MSCs

The 3 sources of aMSCs were purchased from commercial sources: ReachBio human bone marrow mesenchymal stem cells – lot number 0090408 (referred later as RB1 – ReachBio LLC, Seattle, WA), Poietics™ human mesenchymal stem cells – lot number 7F3675 (referred later as PL2 – Lonza Walkersville Inc., Gaithersburg, MD), and Poietics™ human mesenchymal stem cells – lot number OF4266 (referred later as PL3 – Lonza Walkersville Inc., Gaithersburg, MD). All aMSCs were cultured in MesenCult® media and maintained at 37°C in a humidified atmosphere containing 5% CO_2. Medium was changed every 3 days, and cells were passaged when reaching 80% of complete confluence.

3.2.2.2. Fetal human MSCs

fMSCs were isolated from fresh human fetal long bone by Mr. LinMyint Nyan, supervised by K.J. Van Vliet (MIT and SMART BioSyM) and J. Chan (SMART and NUS-Duke and KK Hospital, Singapore) as described in [165] [166] [95]. Fetal tissue collection for research purposes was approved by the Domain Specific Review Board of National University Hospital, in compliance with international guidelines regarding the use of fetal tissue for research [167]. Women who voluntarily terminate their pregnancy gave written consent for the clinical procedure and for the use of fetal tissue for research purposes. The bone marrow was taken from the fetuses' femur, and the cell suspensions were plated to isolate the fMSCs. Those fetal

cells were then frozen using the protocol described in Appendix A. Frozen samples were sent from the National University of Singapore to the Massachusetts Institute of Technology, under dry ice. Upon reception, they were stored for 24h at -80°C, and then in liquid nitrogen. The thawing procedure is described in Appendix A. All fMSCs were cultured in our homemade fetal media (90% Gibco® low-glucose Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) and 10% FBS (lot number 696409, Invitrogen, Carlsbad, CA) and maintained at 37°C in a humidified atmosphere containing 5% CO_2. Medium was changed every 3 days, and cells were passaged when reaching 80% of complete confluence.

3.2.3. Culture protocols, Observation and analysis

All cell sources (the three aMSCs and the three fMSCs described above) were cultured according to protocols described in Appendix A. Experiments were performed in triplicate. The cells were seeded at 500 cells/cm^2 onto Nunc T175 tissue-culture-treated polystyrene flasks. Twenty-four hours after thawing, the culture medium was exchanged and replaced by the same type of fresh medium. Every 3 days, the culture media were exchanged and replaced by fresh medium. Cells were passaged onto new Nunc flasks and seeded at 500 cells/cm^2 when they reached 80% of complete confluence.
Cells were detached from the dish using Trypsin EDTA 1X (0.05% Trypsin/0.53mM EDTA in HBSS without sodium bicarbonate calcium and magnesium – MediaTech Inc., Manassas VA – referred to hereafter as T/E) for 5 minutes at 37°C, and re-suspended in warmed medium. The cells were then run through the spiral channel describe in § 3.2.1

(The protocol to prepare the device and run the cells through is described in Appendix B). Cells were then imaged with an optical microscope in phase contrast (Olympus IX-81). Cell areas were analyzed using the software ImageJ (NIH); this software was used to measure the 'diameter' of the cell. The 'diameter' is the average between the minor and major axis of the fitted ellipse. The experiments were designed to simply separate cells by diameter and analyze the diameter distribution of the subpopulation of aMSCs.

3.3.Results

3.3.1. Diameter distribution of adult and fetal mesenchymal stem cells

The experiments were performed in triplicate for the three aMSC sources mentioned above. For each source, we study the cells at three passages (P3, P6 and P9) to quantify any evolution in MSCs' diameter with aging. Two hundred cells were analyzed for each condition – i.e., 200 cells were measured for RB1 at P3, 200 cells for RB1 at P6, 200 cells for RB1 at P9, 200 cells for PL2 at P3, 200 cells for PL2 at P6, etc.

We found an increase in aMSC average diameter over time for all adult sources. The data quantifying the change in aMSCs diameter distribution with increasing passage number are summarized in Figure 4.

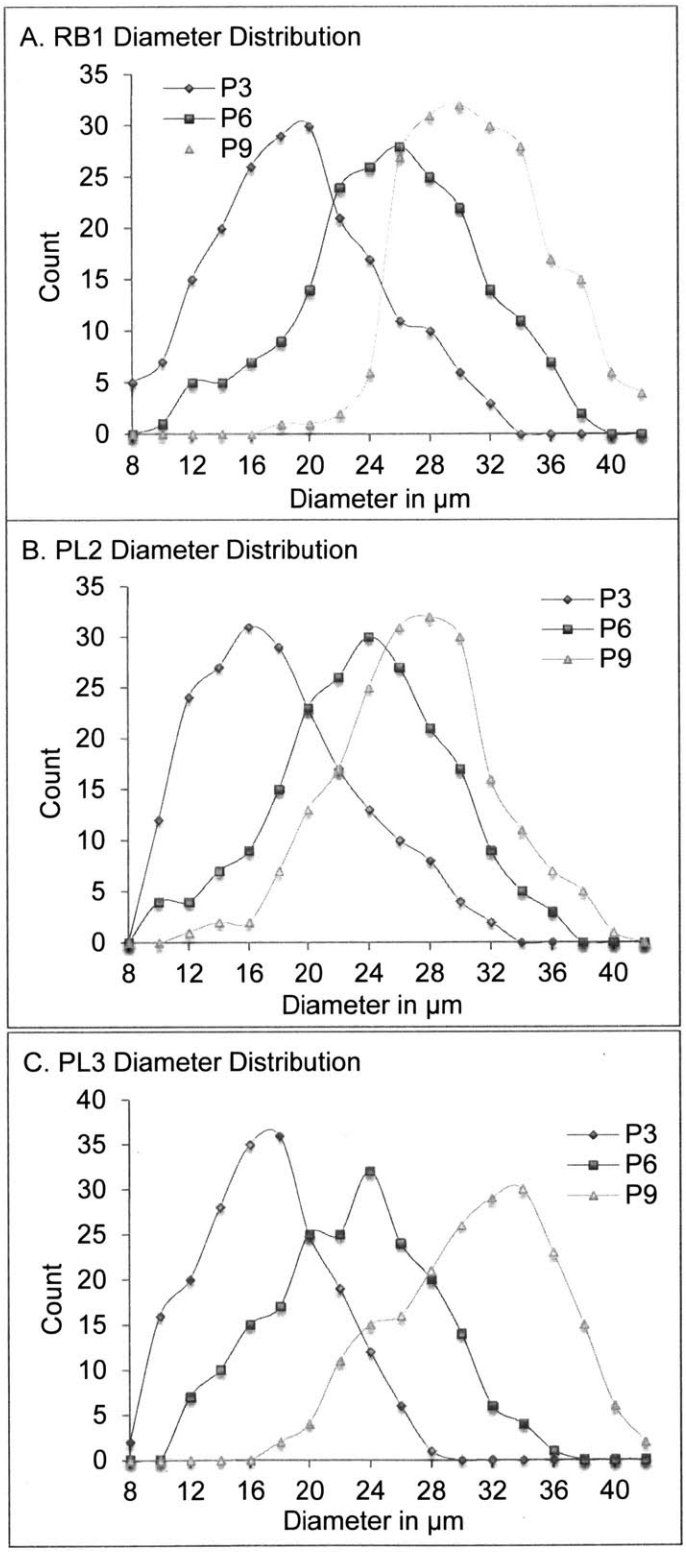
We use the number of cells observed within each bin of cell diameter, or the "count", to compare the distributions; we analyzed the same number of cells for each condition. It is therefore equivalent to graph the frequency of observation, or the percentage of cells within the

measured population with the diameter X, and the number of cell we count with diameter X. The data quantifying the change in aMSCs diameter distribution with increasing passage number are reported in Table 4.

Table 4: Average diameter of aMSCs in function of passage number

Source	Passage #	Average Diameter [µm]	Standard Deviation [µm]
RB1	P3	19.1	2.01
	P6	25.42	2.34
	P9	31.29	2.97
PL2	P3	18.09	1.95
	P6	23.59	2.30
	P9	27.01	2.59
PL3	P3	17.03	2.12
	P6	22.78	2.27
	P9	30.86	2.79

Figure 4: aMSCs' diameter evolution with passaging.



Each dot on the graphs indicates how many cells we measured with a diameter between x and x+2 µm. For example, the dot at 8 µm includes all cells with a diameter ≤ 8 µm. The dot at 20 µm includes all cells verifying 18 µm < diameter ≤ 20 µm. The dots are connected using Microsoft Excel Smooth Marked Scatter function.

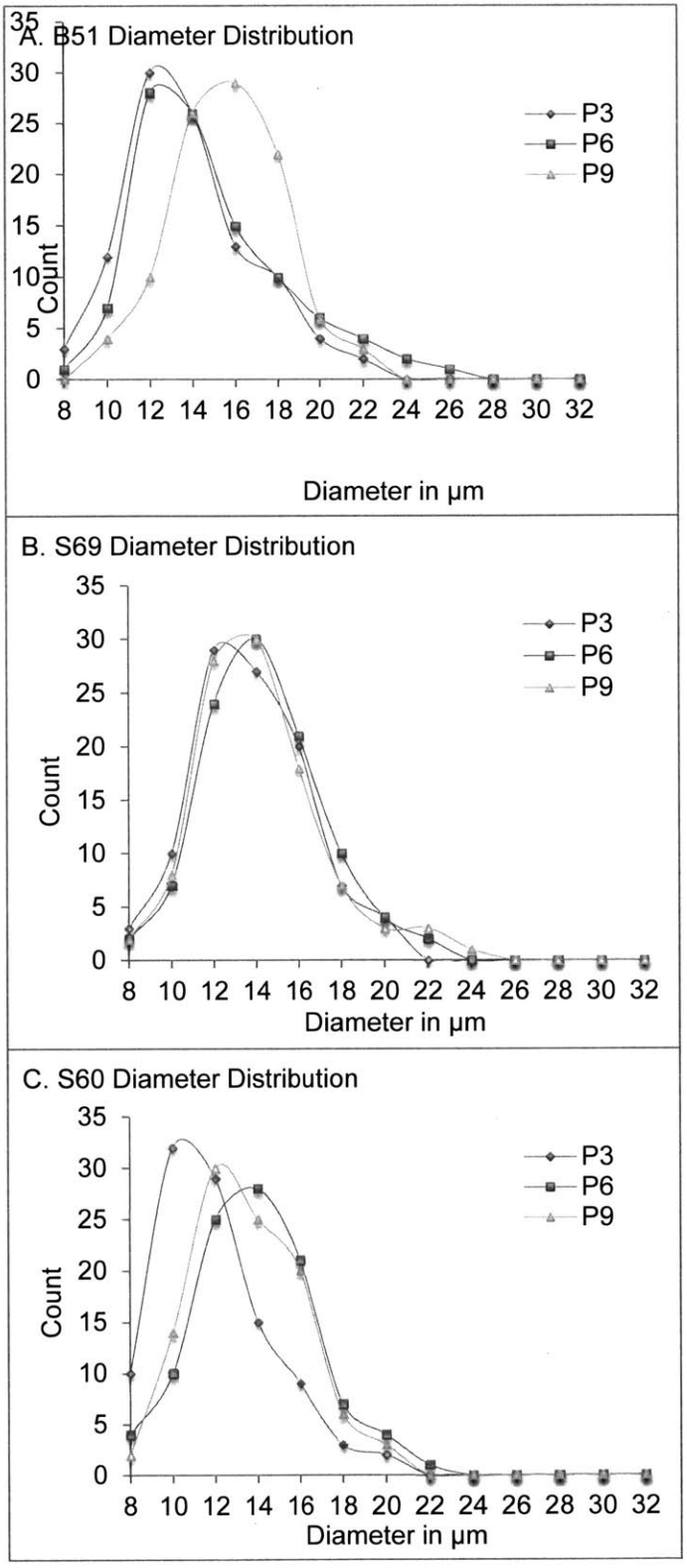
(A) (B) (C) Respectively RB1, PL2 and PL3 aMSCs' average size increases with passaging. The broadness of the distribution is also increasing for RB1, PL2, and PL3 aMSCs with passaging. All aMSC size average and distribution were not increasing and broadening equally due to "patient-to-patient" variations. This confirms our choice to study more than one MSC sources to study size distribution.

We see from Table 3 that the average diameter and standard deviation for all adult sources increased with increasing passage number over three to nine passages. We therefore conclude that the aMSC average diameter is increasing over time, and that the broadness of this distribution – a measure of the heterogeneity of cell diameter – also increases with increasing passage number. However, these data cannot explain how the average cell diameter of the population is increasing. Another set of experiments would be necessary to resolve this issue.

We performed in triplicate the same set of experiments for fMSCs: three fetal sources (named B51, S60 and S69) at three passages (P3, P6 and P9). 200 cells were analyzed for each condition – i.e., 200 cells were measured for B51 at P3, 200 cells for B51 at P6, 200 cells for B51 at P9, 200 cells for S60 at P3, 200 cells for S60 at P6, etc.

We found an almost constant diameter distribution over time for all fetal sources. The data for fMSCs diameter distribution evolution with aging is summarized in Figure 5.

Figure 5: fMSCs' size evolution with passaging.



Each dot on the graphs indicates how many cells we measured with a diameter between x and x+2 µm. For example, the dot at 8 µm includes all cells with a diameter ≤ 8 µm. The dot at 20 µm includes all cells verifying 18 µm < diameter ≤ 20 µm. The dots are connected using Microsoft Excel Smooth Marked Scatter function.

(A) (B) (C) Respectively B51, S69 and S60 fMSCs' average size and broadness of the distribution stay constant with passaging.

Once again, we use the "count" to compare the distributions, as we analyzed the same number of cells for each condition. It is therefore equivalent to graph the percentage of cell within the measured population with the diameter X, and the number of cell we count with diameter X. The data quantifying the change in fMSCs diameter distribution with increasing passage number are reported in Table 5.

Table 5: Average diameter of fMSCs in function of passage number

Source	Passage #	Average Diameter [µm]	Standard Deviation [µm]
B51	P3	13.8	1.34
	P6	14.8	1.28
	P9	15.7	1.71
S69	P3	13.76	1.45
	P6	14.34	1.47
	P9	14.16	1.44
S60	P3	11.96	1.26
	P6	13.88	1.42
	P9	13.54	1.44

We see from Table 5 that the average diameter and standard deviation for all fetal sources seems invariant with increasing passage number. We therefore conclude that the fMSC average diameter as well as the broadness of the distribution is constant over time in culture, at least over this duration and under these *in vitro* conditions.

3.3.2. Comparison of diameter distribution between adult stem cells subpopulation and fetal stem cells

Following the first set of experiments confirming the broadness of the aMSCs diameter distribution. We use the spiral microfluidic device described in §3.2.1 to separate the aMSCs by diameter and analyzed the subpopulation following the protocols described in §3.2.3. As the fMSC population is comparably homogeneous in cell diameter, and we ourselves demonstrated this size distribution to be narrow, we did not separate fMSCs as a function of cell diameter using this device.

We performed in triplicate the same set of experiments for sorted aMSCs as we did in §3.2.3. Two hundred cells were analyzed for each conditions – i.e., 200 cells were measured for RB1 Outlet 4 at P3, 200 cells for RB1 Outlet 3 at P3, 200 cells for RB1 Outlet 2 at P3, 200 cells for RB1 Outlet 1 at P3, 200 cells for RB1 Outlet 4 at P6, 200 cells for RB1 Outlet 3 at P6, etc. We will refer hereafter to Outlet 1 cells as O1 cells, Outlet 2 cells as O2 cells, etc.

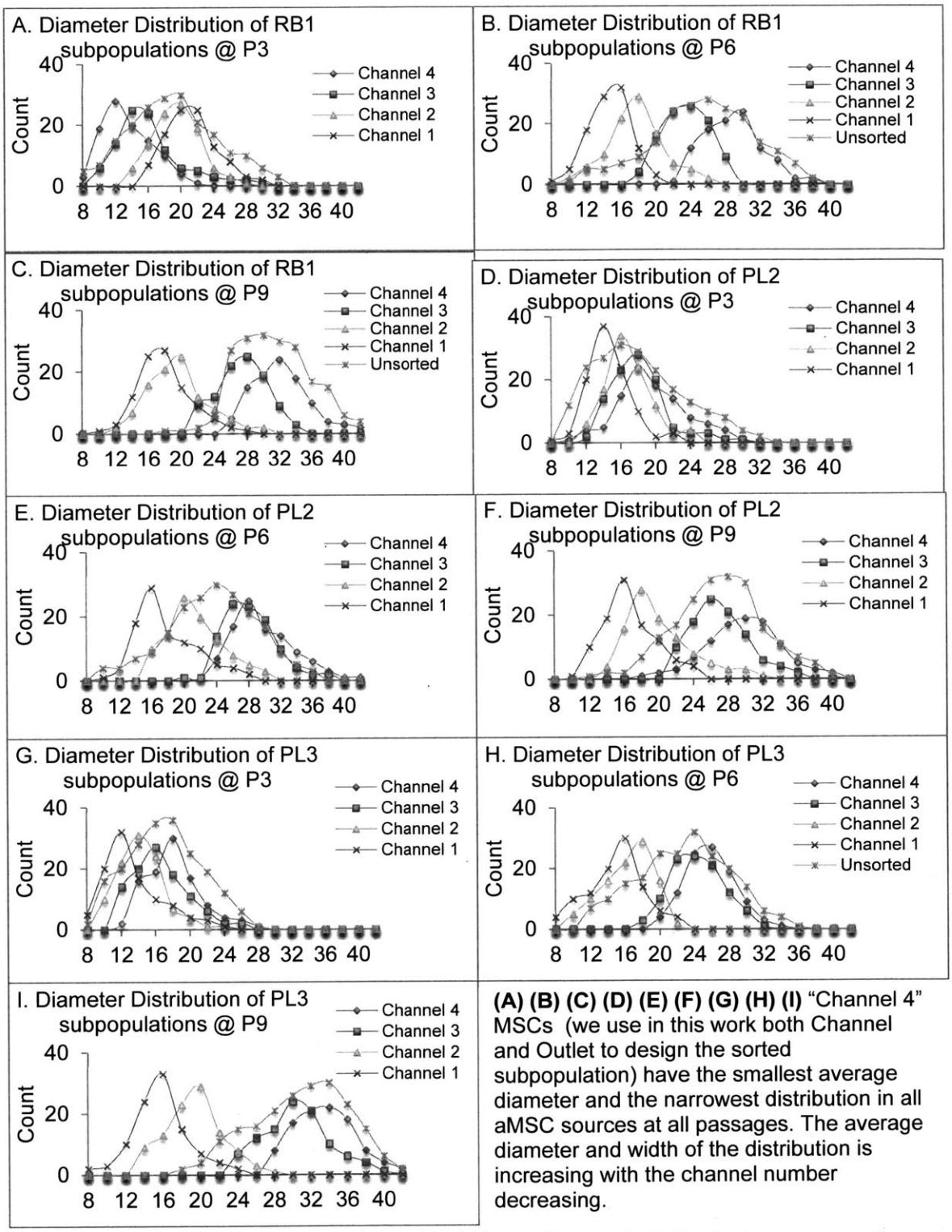
The aMSCs were successfully separated on the diameter basis and we found that all four "Outlet 4"††† aMSCs diameter distribution was close to the diameter distribution of fMSCs in both average diameter and standard deviation. These data are shown in Table 5.

We efficiently separated aMSCs by diameter via this approach. For example, "unsorted" RB1 aMSCs at P3 had an average diameter at P3 of 19.1 ± 2.01 μm, whereas "O1" RB1 aMSCs at P3 had an average diameter of 21.32 ± 1.81μm and "O4" RB1 aMSCs at P3 had an average

††† "Outlet 4" means the smaller subpopulation of aMSCs that we isolated using the spiral microfluidics device. We called it "Outlet 4" before they are coming from the outlet number 4 of this device (confer Figure 3)

diameter of 13.52 ± 1.15μm. In this study, "unsorted" aMSCs mean that the cells were not run through the device, and "sorted" aMSCs mean that the cells were isolated into subpopulations through the device. In all cases and at all passages, "O4" aMSCs were smaller than those collected from the other channels, especially cells collected from "O1" and "O2". These data are shown in Figure 6.

Figure 6: "Unsorted" and "Sorted" aMSCs' diameter evolution with passaging.



(A) (B) (C) (D) (E) (F) (G) (H) (I) "Channel 4" MSCs (we use in this work both Channel and Outlet to design the sorted subpopulation) have the smallest average diameter and the narrowest distribution in all aMSC sources at all passages. The average diameter and width of the distribution is increasing with the channel number decreasing.

All horizontal axes are the diameter in μm. Each dot on the graphs indicates how many cells we measured with a diameter between x and x+2 μm. For example, the dot at 8 μm includes all cells with a diameter ≤ 8 μm. The dot at 20 μm includes all cells, verifying 18 μm < diameter ≤ 20 μm. The dots are connected using Microsoft Excel Smooth Marked Scatter function. All horizontal axes are diameter of the cell in μm.

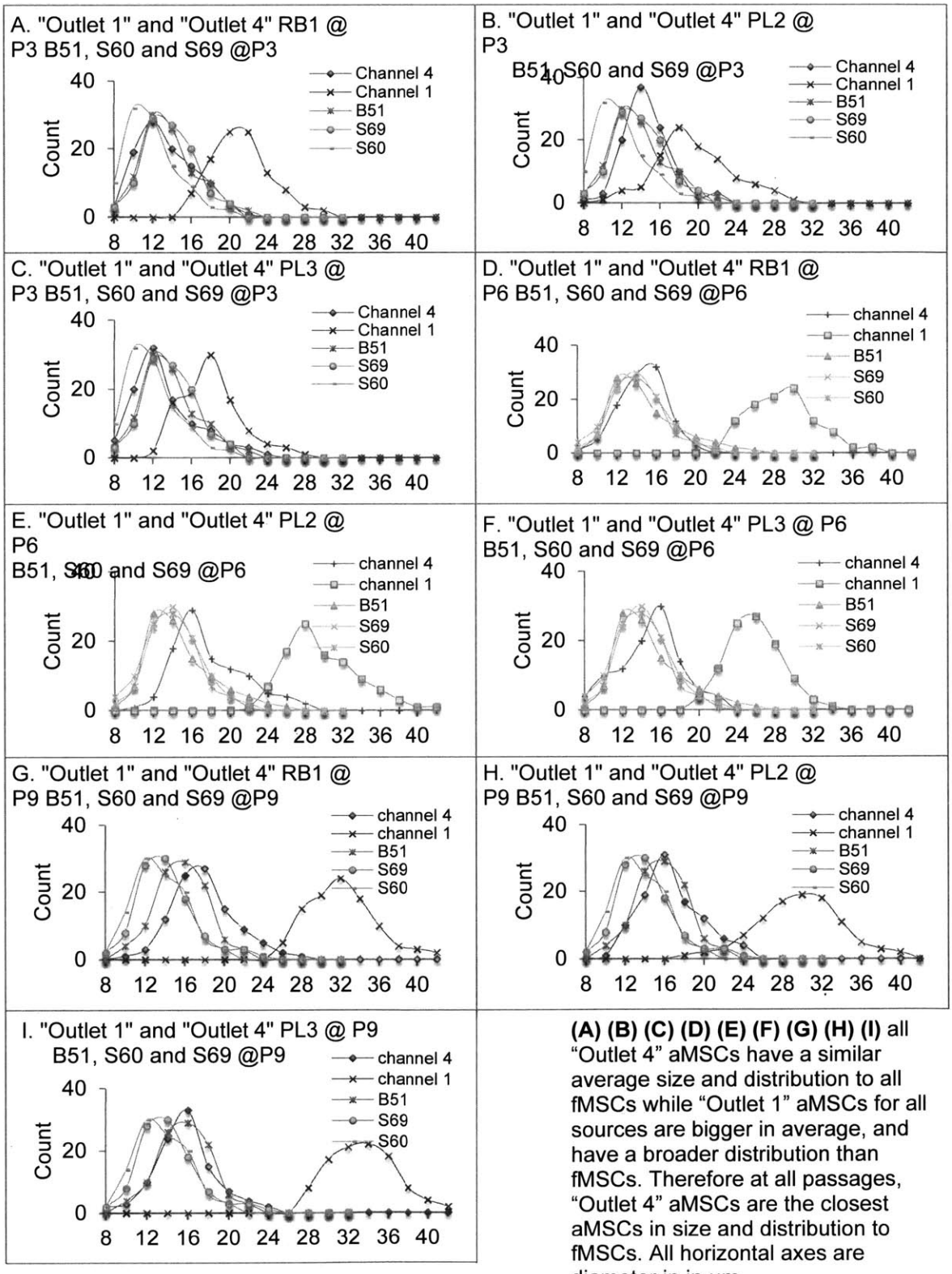
Table 6: Average diameter of "sorted" aMSCs in function of passage number.

Source	Passage #	Outlet #	Average Diameter [µm]	Standard Deviation [µm]
RB1	P3	Outlet4	13.52	1.15
		Outlet3	16.18	1.19
		Outlet2	19.48	1.76
		Outlet1	21.32	1.81
	P6	Outlet4	14.68	1.55
		Outlet3	17.72	1.54
		Outlet2	23.42	1.21
		Outlet1	28.9	1.35
	P9	Outlet4	17.98	1.52
		Outlet3	19.58	1.51
		Outlet2	27.4	1.36
		Outlet1	32.16	1.52
PL2	P3	Outlet4	14.66	1.52
		Outlet3	16.88	1.64
		Outlet2	18.1	1.59
		Outlet1	19.58	1.41
	P6	Outlet4	17.84	1.35
		Outlet3	21.4	1.67
		Outlet2	28.2	1.34
		Outlet1	29.78	2.18
	P9	Outlet4	16.66	1.44
		Outlet3	20.1	1.52
		Outlet2	27.1	2.21
		Outlet1	29.74	2.05
PL3	P3	Outlet4	13.36	1.10
		Outlet3	14.34	1.38
		Outlet2	16.56	1.36
		Outlet1	18.1	1.56
	P6	Outlet4	14.96	1.33
		Outlet3	16.32	1.57
		Outlet2	24.3	2.10
		Outlet1	25.78	2.33
	P9	Outlet4	15.76	1.48
		Outlet3	19.34	1.71
		Outlet2	30.4	2.36
		Outlet1	33.5	2.70

As we showed with figure 4, 5 and 6 "O4" aMSCs comprise the smallest and most narrowly distributed subpopulation of aMSCs, and "O1" aMSCs comprise the largest and most broadly distributed. Therefore, to simplify the figures shown in this work, we only graphed the diameter distribution of "O4" aMSCs, "O1" aMSCs, and fMSCs. We found that "O4" aMSCs were very similar to fMSCs in both average diameter and broadness of distribution. "O1" aMSCs exhibited a broader range of diameters with an average of 26.54 ± 2.1μm clearly larger than that of fMSCs. The results are shown in Figure 7.

We conclude from the data shown in Figures 7 that fMSCs at all passages are similar in average size and distribution to "O4" aMSCs at all passages. Therefore, even if the overall population of aMSCs is increasing in size over time, we are still able to separate aMSCs by size to find a subpopulation of aMSCs with similar size profile to fMSCs.

Figure 7: "Outlet 4", "Outlet 1" aMSCs size distributions in comparison to fMSCs size distribution at passage P3, P6 and P9.



(A) (B) (C) (D) (E) (F) (G) (H) (I) all "Outlet 4" aMSCs have a similar average size and distribution to all fMSCs while "Outlet 1" aMSCs for all sources are bigger in average, and have a broader distribution than fMSCs. Therefore at all passages, "Outlet 4" aMSCs are the closest aMSCs in size and distribution to fMSCs. All horizontal axes are diameter in in μm.

3.4.Outcomes

3.4.1. Conclusions

The goal of this study was to isolate a subpopulation of aMSCs similar in size to fMSCs. Lee *et al.* already fractionated cell subpopulations as a function of diameter and demonstrated cell cycle synchronization and using the same device [110]. Using this spiral microfluidics device, we were able to reproduce Lee's data and to separate aMSCs by size with a sufficient resolution to clearly isolate subpopulations with different average sizes and distribution. We found that "O4" aMSCs had the closest average size and distribution to fMSCs'. However, the distribution of "O4" aMSCs is still broader and the mean cell diameter is sometimes larger than the distribution and the mean cell diameter of fMSCs.

We also conclude from this work that the average diameter of the aMSCs population average size and distribution is increasing with passaging. This conclusion is true for O1, O4 and unsorted aMSCs. In contrast, the mean diameter of the fMSC population is relatively constant with increasing passage number. This suggests that, if one's goal is to obtain a subpopulation of aMSCs that is most similar in diameter to that of fMSCs, this can most easily be achieved by minimizing the in vitro passaging numbers of aMSCs.

3.4.2. Possibilities for continued research

There are many appealing directions along which to continue this research. First, the study could be extended to incorporate cells obtained from more patient sources. Cell sources are variable and it is thus good to confirm our results with more experiments.

Second, the obvious continued research (and the one we partly address in this work) is to study if those "O4" cells are similar to fMSCs in terms of other properties more functionally useful than cell diameter. The list of properties to study can be extended to many more properties than the one we will study in this work, and include *in vivo* differentiation potentials, mobilization‡‡‡ capacities, and ultimately, the therapeutic potential.

Third, it would be interesting to study the shift of the aMSC populations' diameter distribution. It would be interesting to know whether this shift to increasing mean diameter with increasing passage number comes from: (1) all individual cells becoming larger with each passage; or (2) only small (or large) cells becoming larger.

Ultimately, it would be of great interested to study if those "O4" cells are better for therapy than the bigger "O1" aMSCs and better than (as at least as good as) fMSCs.

‡‡‡ mobilization is the capacity of MSCs to differentiate into different lineages in distant tissues

Chapter 4. Cell migration velocity

4.1. Study background, hypothesis and design

As mentioned in §3.1: fMSCs derived from fetal bone marrow are more plastic and grow faster than aMSCs [53] and are therefore of great interest for stem cell-based therapy. aMSCs present some interesting practical and ethical advantages and fMSCs face some practical and ethical issues. Hypothesizing that some aMSCs could combine both advantages, we isolated in the previous chapter a subpopulation of aMSCs with similar average size and distribution to fMSCs.

As reported in murine [168], simian [169] [170] [171], and human [172] models, MSCs are able to colonize and persist long-term in a wide range of tissues. The factors that guide MSCs to appropriate microenvironments or induce their circulation are yet to be fully understood, but Lopez-Ponte *et al.* demonstrated that the *in vitro* migration capacity of MSCs is linked to their sensitivity to the inflammatory cytokines interleukin 1β and tumor necrosis factor α [173]. Stem cell-based therapy lies on these MSCs' abilities to colonize, differentiate and persist long term in these microenvironments. Therefore, studying the migration capacity of our sorted aMSCs and fMSCs could answer one aspect of the last question motivated in Chapter 3: Are "Outlet 4" aMSCs better for therapy than the larger "Outlet 1" aMSCs, and better than (or at least as good as) fMSCs?

Cell migration characteristics have been well studied over recent years, and fluorescent dyes were increasingly exploited to track migration and proliferation; this enhanced image contrast facilitates automated image analysis. This is especially true for MSCs, as they have a well-spread

morphology [3] [2] and are therefore difficult to track when imaged with an optical microscope in phase contrast. We therefore chose to use a fluorescent dye such as CellTracker™ Green CMFDA (5-Chloromethylfluorescein Diacetate) (Molecular Probes®) to address this issue. However, Parish *et al.* showed that all dyes are not equivalent for lymphocyte migration and proliferation [174] and De Clerck *et al.* showed that fluorescent dyes could interfere with some cellular functions [175]. Therefore, we first studied in this work the influence of CellTracker™ Green CMFDA on MSC migration velocity, in order to determine if using this dye to study MSC migration velocity would influence our results quantitatively.

Second, we know that migration of cells is mediated by several factors including extracellular matrix-adhesion receptors such as integrins [176]. Integrins link the cell to extracellular matrix ligands [177] [178] and this link affects cells migration velocity [179] [180]. Palecek *et al.* [181] showed that the cell speed is dependent on fibronectin coating concentration. Therefore, we also determined the amount of fibronectin that maximized cell migration velocity for our experiment.

At last, and to continue to contribute answers to the question posed in Chapter 3, we studied the migration capacity of fMSCs, and compared the migration capacities of those fMSCs with that of "size-sorted" aMSCs subpopulations.

4.2.Materials and Methods

4.2.1. Establishment of MSCs culture

The 3 sources of aMSCs were purchased from vendors: ReachBio human bone marrow mesenchymal stem cells – lot number 0090408 (referred later as RB1 – ReachBio LLC, Seattle, WA), Poietics™ human mesenchymal stem cells – lot number 7F3675 (referred later as PL2 – Lonza Walkersville Inc., Gaithersburg, MD), and Poietics™ human mesenchymal stem cells – lot number OF4266 (referred later as PL3 – Lonza Walkersville Inc., Gaithersburg, MD). All aMSCs were cultured in MesenCult® media or in our homemade media and maintained at 37°C in a humidified atmosphere containing 5% CO_2. Medium was changed every 3 days, and cells were passaged when reaching 80% of complete confluence.

fMSCs were isolated from fresh human fetal long bone as described in Chapter 3. Upon receipt of frozen cells at MIT, they were stored for 24h at -80°C, and then in liquid nitrogen. The thawing procedure is described in Appendix A. All fetal hMSCs were cultured in our homemade fetal media (90% Gibco® low-glucose Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) and 10% FBS (lot number 696409, Invitrogen, Carlsbad, CA) and maintained at 37°C in a humidified atmosphere containing 5% CO_2. Medium was changed every 3 days, and cells were passaged when reaching 80% of complete confluence.

4.2.2. Preparation and characterization of media and plastics

MesenCult® medium (90% MesenCult® basal media plus 10% MesenCult® supplements) was used for aMSCs, and our homemade medium (90% Gibco® low-glucose Dulbecco's modified Eagle's medium - Invitrogen, Carlsbad, CA plus 10% selected lot FBS - lot number 696409) was used for fMSCs. Elements of both media were mixed at room temperature and filtered using Nalgene MF75™ Series filter (Product # 295-3345 - Thermo Fisher Scientific, Rochester, NY). All cells were seeded on Nunc T175.

4.2.3. Cell Culture, observation and analysis

All cells were cultured according to protocols described in Appendix A. Experiments were performed in triplicate. The cells were seeded at 500 cells/cm^2 onto Nunc T175. Twenty-four hours after thawing, the culture medium was exchanged and replaced by fresh medium. Every 3 days, the culture medium was exchanged and replaced by fresh medium. When reaching 80% of complete confluence, cells were detached from the dish using T/E for 5 minutes at 37°C, re-suspended in medium, and centrifuged at 1200 rpm for 8 minutes. Then the cells were re-seeded onto new flasks at 500 cells/cm^2.

To study cell migration velocity, we seeded MSCs (after detaching them using T/E) at 100 cells/cm^2 on a fibronectin-coated 35 mm glass bottom Petri dish (Product # D35-20-0-N – In Vitro Scientific, Sunnyvale, CA). The dishes were functionalized for cell attachment by adding a fibronectin solution (1 mg/mL –in phosphate buffered saline; SIGMA F2006 Fibronectin from human plasma, Sigma-Aldrich, St. Louis, MO) following the protocol described at http://www.invitrosci.com/. The Petri dish was incubated for one hour at 37°C, 5% CO_2. The dish was then rinsed with purified water.

The author then seeded the cells onto the dish at 100 cells/cm^2 and let the MSCs attached for two hours. Cells were then imaged every 5 minutes for 8 hours with an optical microscope in phase contrast (Olympus IX-81). Cell migration paths were analyzed using the software Metamorph® for Olympus (Molecular Devices, Sunnyvale, CA); the author used this software to extract cell center of mass, and compute cell velocity for each time step. The author then averaged these velocities to find the average velocity of each cells.
This study is separated in three sub-tasks: (1) Quantify the influence of CellTracker® on cell migration velocity; (2) Determine the ideal coating concentration of fibronectin to maximize the average velocity of the MSCs; and (3) Compare migration capacities of "size-sorted" aMSCs and fMSCs.

4.3. Impact of Cell Tracker®

As mentioned in § 4.1, aMSCs are difficult to track when imaged with an optical microscope in phase contrast. To help with this issue and make tracking easier and faster, we decided to use CellTracker™ Green CMFDA. As De Clerck *et al.* [175] showed that fluorescent dyes could interfere with some cellular functions, we studied the influence of this fluorescent dye on aMSC migration velocity.

Molecular Probes® CellTracker™ fluorescent dye pass freely through cell membranes and are converted to cell-impermeant reaction products that can be passed to daughter cells through several divisions. When using CellTracker™, cells are fluorescent and viable for at least 24 hours. The signal from CellTracker™ dyes is monitored using fluorescence microscopy (http://products.invitrogen.com/ivgn/product/C2925).

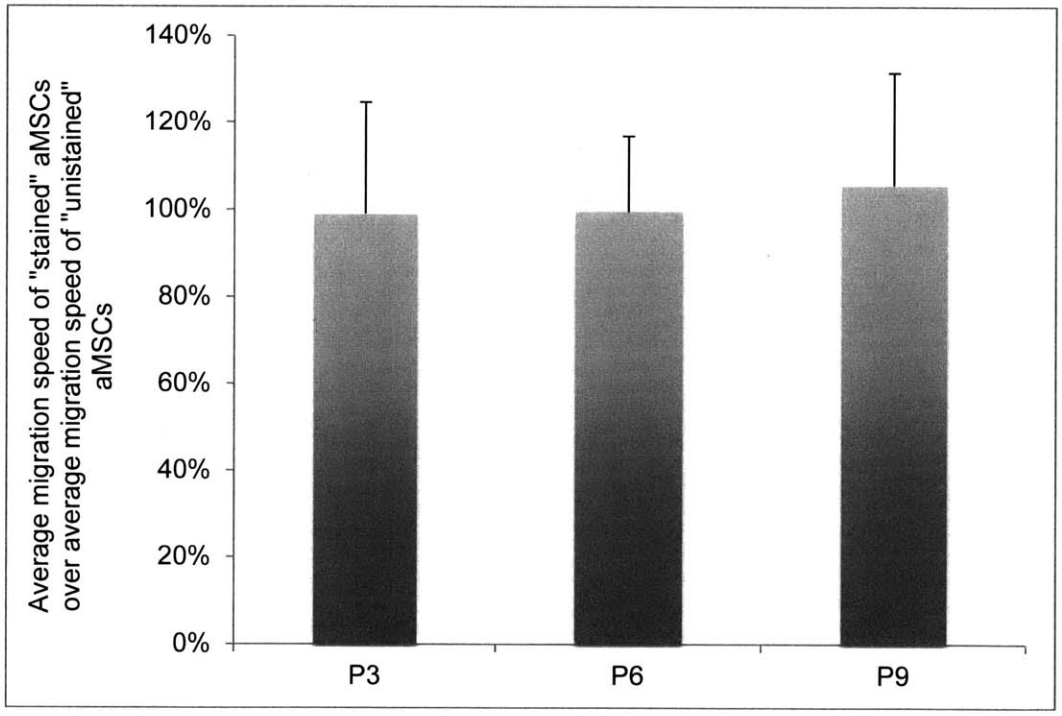
We mentioned in § 4.1 that the coating concentration of fibronectin could influence the maximal speed of migration. We will address this concern in § 4.4. Therefore for this study, we coated the glass-bottom Petri dish with the concentration of 75 μg/mL that is often reported in the literature for such studies of cell migration [179]. The RB1 aMSCs were seeded on Petri Dish at 100 cells/cm^2 using the protocol described in § 4.2.3.

To study the effect of CellTracker™ Green CMFDA (referred to hereafter as CellTracker), we imaged aMSCs RB1 at three different passages (P3, P6, and P9) with and without the dye. We acquired an image every five minutes for eight hours with an optical microscope using fluorescence filter (Semrock GFP-3035B [EX457/487 EM502/538]) when the MSCs were stained with CellTracker, and in phase contrast when MSCs were not stained (Olympus IX-81). We followed the protocol given by Molecular Probes® to stain the aMSCs (http://tools.invitrogen.com/content/sfs/manuals/mp02925.pdf - described in Appendix C).

The experiments were performed in triplicate and 200 cells were analyzed for each condition – i.e., 200 cells were measured at P3 for "stained" aMSCs, 200 cells at P6 for "stained" MSCs, 200 cells at P9 for "stained" aMSCs, 200 cells at P3 for "unstained" aMSCs, 200 cells at P6 for "unstained" aMSCs and 200 cells at P9 for "unstained" aMSCs.
We did not find any significant difference in average migration velocity between "stained" and "unstained" aMSCs, and therefore conclude that CellTracker™ Green CMFDA can be used to study migration velocity of MSCs as a function of other variables (e.g., patient source). To make sure there was no statistically significant difference, we used a two-tailed t-test to determine if the "stained " and "unstained" aMSCs had a statistically significantly different ($p > 0.5$) average migration velocity. The data for these experiments are summarized in Figure 8. We note that we did not quantify other aspects of migration behavior (e.g., persistence time or

length, protrusion dynamics), so it remains possible and beyond the scope of this study whether CellTracker affects other characteristics of cell migration appreciably.

Figure 8: Average migration velocity of CellTracker™ Green CMFDA "stained" over average migration velocity of "unstained" aMSCs at 3 different passages.



The error bars are geometric standard deviation. Stained aMSCs are as fast as unstained aMSCs at every passage.We used a two-tailed t-test to determine if the aMSCs at different passages had a statistically significantly different ($p > 0.5$) average migration velocity.

4.4.Results

Despite the results of the previous experiment, all experiments related to migration will be performed with "unstained" cells. This approach could seem tedious, but at the time of these studies, the author had issues with using correctly CellTracker™ Green CMFDA and completed the migration capacity analysis before succeeding in staining the MSCs properly.

Migration of cells is mediated by several factors including adhesion receptors such as integrins [176]. Integrins link the cell to extracellular matrix ligands [177] [178] and this affects cells migration velocity [179] [180]. Palecek *et al.* [181] showed that the cell speed is dependent on fibronectin coating concentration. This is why we first considered the ideal amount of coating concentration of fibronectin.

To determine this ideal amount of coating, we coated our Petri dishes (vendor) following the protocols described in § 4.1 with five different fibronectin concentrations: 10, 20, 50, 75 and 100 µg/mL. The RB1 aMSCs were seeded on Petri dishes at 100 cells/cm^2 using the protocol described in § 4.2.3.

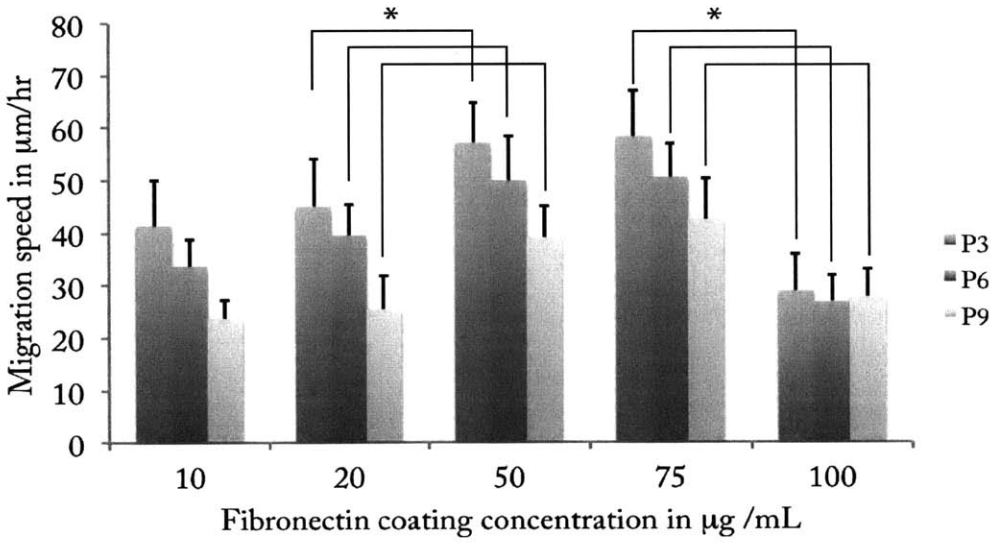
To study the effect of fibronectin coating concentration, we imaged aMSCs RB1 at 3 different passages (P3, P6, and P9) onto five different Petri dishes coated with the five concentrations of fibronectin. We then took an image every five minutes for eight hours with an optical in phase contrast (Olympus IX-81).

The experiments were performed in triplicate and 100 cells were analyzed for each condition – i.e., 100 cells were measured for RB1 at P3 onto dish coated with 10 µg/mL of fibronectin, 100 cells were measured for RB1 at P6 onto dish coated with 10 µg/mL of fibronectin, 100 cells were measured for

RB1 at P9 onto dish coated with 10 μg/mL of fibronectin, 100 cells were measured for RB1 at P3 onto dish coated with 20 μg/mL of fibronectin, 100 cells were measured for RB1 at P6 onto dish coated with 20 μg/mL of fibronectin, etc. We found a biphasic behavior with a maximum cell velocity for a fibronectin concentration of 75 μg/mL. These data are shown in Figure 9.

Figure 9: RB1 aMSCs average velocity as a function of fibronectin coating concentration at different passages.

The error bars are geometric standard deviation. We also noticed from this experiment that P3 aMSCs are faster than P6 aMSCs that are faster than P9 aMSCs for all fibronectin coating concentration studied, except for the highest concentration of 100 μg/mL. We used a two-tailed t-test to determine if the aMSCs at one passage and at different coating concentration had a statistically significantly different (* $p < 0.05$) average migration velocity.



The biphasic behavior is not surprising, as biphasic dependence of cell migration speed on cell-substratum adhesiveness has been predicted theoretically and proved experimentally for MSCs [182] [181]. We therefore here confirmed for MSCs that migration speed depends in biphasic manner on attachment strength, with maximal migration at an intermediate level of cell-substratum adhesiveness. To compare migration capacities of "size-sorted" aMSCs and fMSCs, we used a fibronectin coating concentration of 50 μg/mL. Even if MSCs did not reach their maximal speed at this concentration, the small difference of speed between 50 and 75 μg/mL leads us to consider the economic component of this study (i.e., fibronectin is expensive) and work with the lower concentrations of fibronectin when possible.

As mentioned in § 4.1, stem cell-based therapy presumes the capacity of MSCs to colonize, differentiate and persist long term in a wide range of microenvironments *in vivo*. As Lopez-Ponte *et al.* demonstrated that the *in vitro* migration capacity of MSCs are linked to their sensitivity to the inflammatory cytokines interleukin 1β and tumor necrosis factor α [173], it is of great interest to study MSCs migration capacity to answer our question: can we find an aMSC that combine both advantages from an fMSC and an aMSC, at least in terms of migration characteristics?

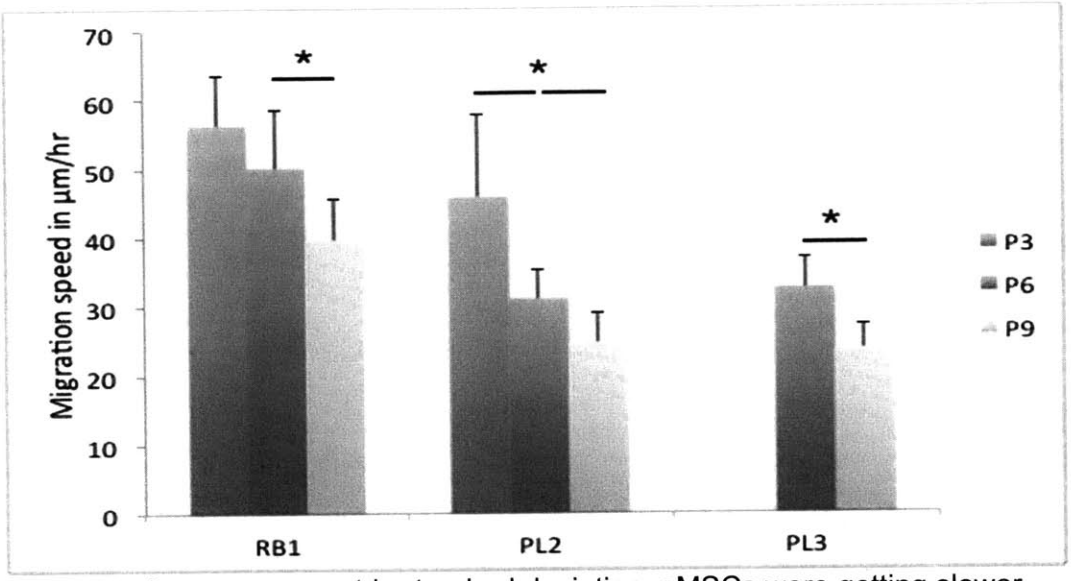
As we previously isolated a subpopulation of aMSCs that are similar in size of fMSCs, we considered whether this or other subpopulations exhibited the same migration capacities as fMSCs. For coherence in our work, and also to try to find an aMSC that combines several properties of an fMSC, we will study the migration capacity of our four adult subpopulations obtained through the spiral microfluidics device. Therefore, we will compare fMSCs' migration velocity with the "O1", "O2", "O3" and "O4" aMSC subpopulations of decreasing average cell diameter.

We used for this study 6 MSCs sources: RB1, PL2, PL3, B51, S60, and S69 at 3 different passages: P3, P6 and P9. We performed our experiments in triplicate following the protocols described in § 4.2.3. 100 cells were analyzed for each condition – i.e., 100 cells were measured for RB1 at P3, 100 cells were measured for RB1 at P6, 100 cells were measured for RB1 at, 100 cells were measured for PL2 at P3, 100 cells were measured for PL2 at P6, etc.

We found that all "O 4" aMSCs were faster than "O1" aMSCs in all experiments. To make sure our results were statistically significant, we used a two-tailed t-test to determine if the subpopulations of aMSCs had a statistically significantly different ($p < 0.05$) average migration velocity. These data are shown in Figure 11. We conclude from the experiments that the fastest aMSCs were "Outlet 4" aMSCs. We don't show the data from "Outlet 3" and "Outlet 2" aMSCs' migration velocity for clarity. However, we found that "Outlet 3" cells were faster than "Outlet 2" aMSCs that were also faster than "Outlet 1" cells. All three subpopulations were slower than "Outlet 4" aMSCs.

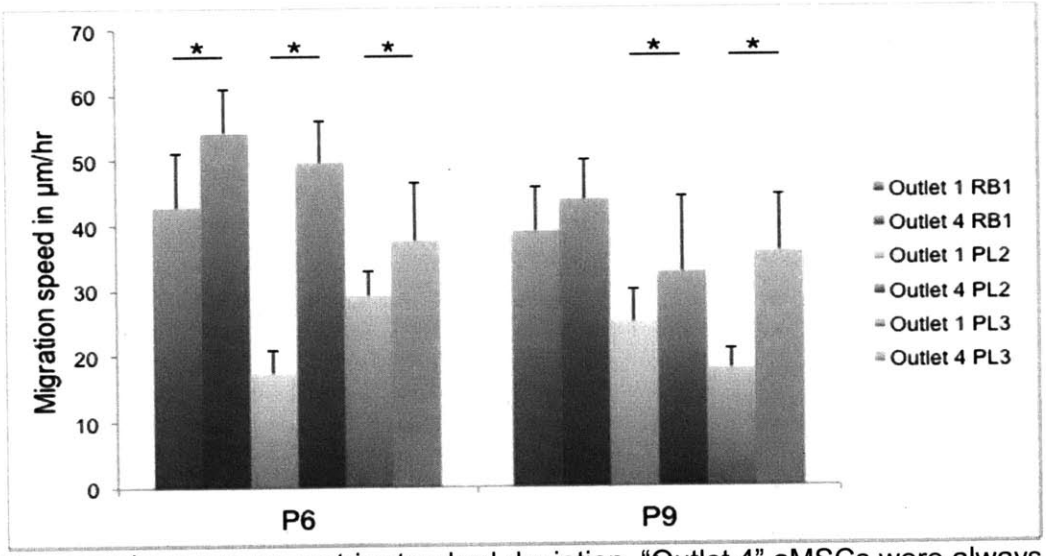
We also found that average speed of aMSCs was decreasing with passages. To make sure our results were statistically significant, we used a two-tailed t-test to determine if the aMSCs at different passages had a statistically significantly different ($* p < 0.05$) average migration speed. These data are shown in Figure 10.

Figure 10: aMSCs migration speed at three passages P3, P6 and P9.



The error bars are geometric standard deviation. aMSCs were getting slower with passages. We used a two-tailed t-test to determine if the aMSCs at different passages had a statistically significantly different (* $p < 0.05$) average migration speed.

Figure 11: aMSCs' subpopulation migration speed at two passages P6 and P9.

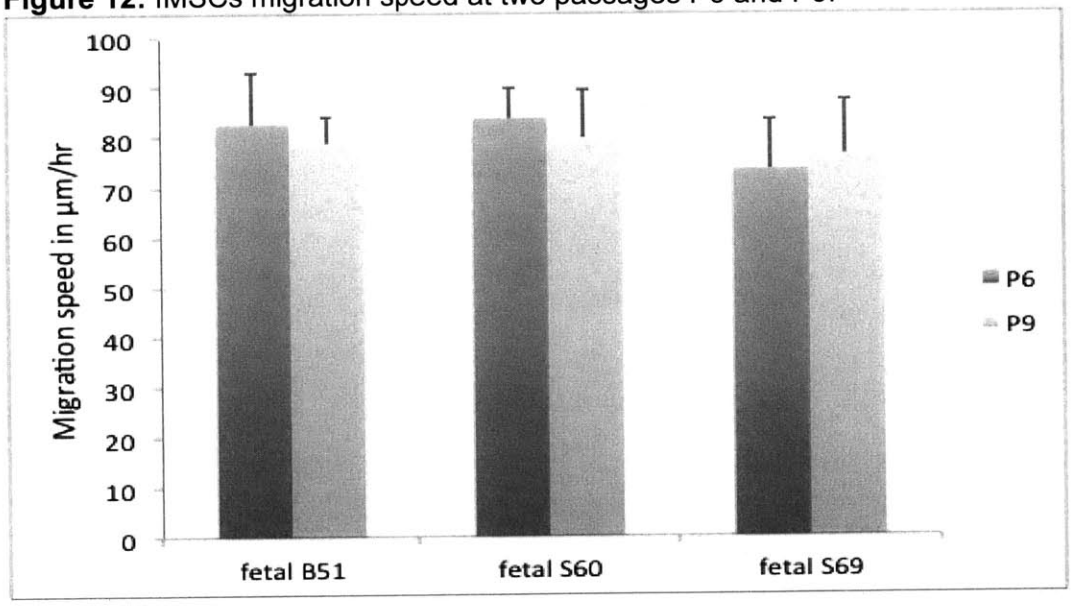


The error bars are geometric standard deviation. "Outlet 4" aMSCs were always faster than "Outlet 1" aMSCs except for RB1 at P9. This could be explained by .an insufficient number of cells analyzed to narrow the distribution of migration velocity. We used a two-tailed t-test to determine if the aMSCs at different passages had a statistically significantly different (*$p < 0.05$) average migration speed.

We determined above that all 4 subpopulations of aMSCs have different migration velocities. To know which of these subpopulations are closer to fMSCs, we need now to compare the migration speed of aMSCs' subpopulations and fMSCs. In order to simplify the figures shown in this work, we only graphed the migration speed of the fastest and the slowest aMSC subpopulations (i.e., "04" and "01" aMSCs, respectively) and fMSCs. Data are shown in Figure 13.

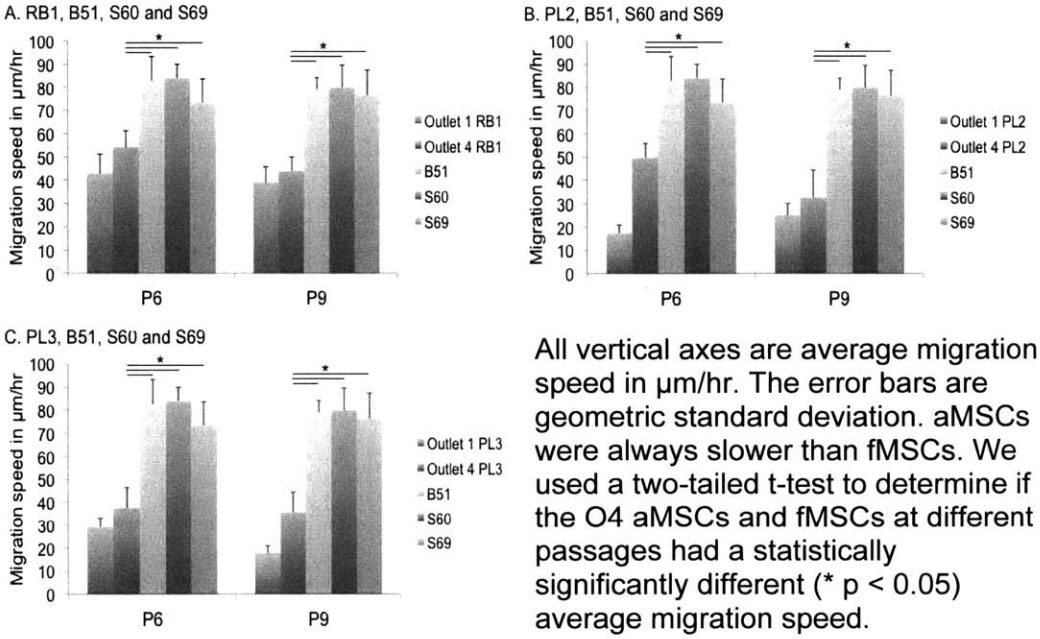
We also found that average speed of fMSCs was not increasing or decreasing with passages. To make sure our results were statistically significant, we used a two-tailed t-test to determine if the aMSCs at different passages had a statistically significantly different ($p > 0.05$) average migration speed. These data are shown in Figure 12.

Figure 12: fMSCs migration speed at two passages P6 and P9.



The error bars are geometric standard deviation. We used a two-tailed t-test to determine if the aMSCs at different passages had a statistically significantly different ($p > 0.05$) average migration speed.

Figure 13: "O4" and "O1" aMSCs migration speeds in comparison to fMSCs migration speed.



All vertical axes are average migration speed in µm/hr. The error bars are geometric standard deviation. aMSCs were always slower than fMSCs. We used a two-tailed t-test to determine if the O4 aMSCs and fMSCs at different passages had a statistically significantly different (* $p < 0.05$) average migration speed.

We found that "O4" aMSCs (42,54 um/hr) were faster than "O1" aMSCs (26.52 um/hr), and slower than fMSCs (77,24 um/hr). We therefore conclude than our subpopulations are not similar in migration velocity to fMSCs, although we identified differences in this velocity among aMSC subpopulations.

4.5.Outcome

4.5.1. Conclusions

This work first aimed to determine the effect of CellTracker™ Green CMFDA on aMSCs migration velocity. We conclude from these experiments that CellTracker™ Green CMFDA has no effect on aMSC migration velocity, and we can therefore use it to facilitate and increasingly automate cell migration tracking.

Then, the author aimed at estimating the optimal fibronectin coating concentration in order to maximize MSC migration speed. We conclude that 75 μg/mL was the optimal coating concentration of fibronectin. However and to conserve costs, we used in our experiments 50 μg/mL as the migration velocity of MSCs was not much slower with this concentration.

We found that average speed of fMSCs was not increasing or decreasing with passages, and that average speed of aMSCs was decreasing with passages.

The primary goal of this study was to identify wheter a subpopulation of aMSCs existed with similar migration velocity to that of fMSCs. Using the 4 subpopulations obtained by size separation, we found that the smallest subpopulation, i.e., "Outlet 4" aMSCs, exhibited migration velocity most similar to that of fMSCs. However, we also found that fMSCs were still much faster than all subpopulations of aMSCs, by 76% under these conditions as compared to O4 aMSCs. Therefore, we found a subpopulation (i.e., "Outlet 4") of aMSCs that is similar in size to fMSCs, and faster than the other aMSCs subpopulations, but still much slower than fMSCs. This could be due to an insufficient resolution to separate our aMSCs as a function of diameter for small diameters. If *in vitro* migration capacity were proven to be the key capacity for "quality" stem cells in clinical settings, tone could refine our sorting approach to attempt to isolate an aMSC subpopulation of maximal migration velocity.

4.5.2. Possibilities for continued research

There are many appealing directions along which to continue this research. First, we only studied migration velocity on fibronectin. Therefore, it could be interesting to study the aMSC's migration capacity on different matrix proteins such as collagen. We could also try to probe in more details the maximum migration velocity as a function of fibronectin coating concentration. This study would give us insights on both the mechanism and the effect of the extracellular matrix molecules on migration.

Second, several fluorescent probes are available. As fluorochrome labeling can interfere with other cellular functions [175], it could be interesting to study (whenever possible) the effect of other fluorescent due on the cell function that is studied.

Third, and in direct connection with the work done in this chapter, it would be of great interest to study other properties of the MSCs' migration. We could reanalyze the present data and new data to quantify other important characteristics of the MSCs' migration behavior (e.g., persistence length and time, total migration distance, protrusion velocity [183]). It would also be interesting to increase the resolution of separation in order to determine if a more refined subpopulation of aMSCs could achieve migration velocities similar to the migration speeds of fMSCs.

Chapter 5. Cell mechanical properties

5.1. Study background, hypothesis and design

As reported by many authors, MSCs have unique mechanical properties, which differ greatly from fully differentiated cells [184]. MSCs have also been reported heterogeneous in mechanical properties [185]. Even if the mechanisms responsible for MSC mechanical properties are not yet fully understood, researchers have found membrane tension, cytoskeleton organization and cytoskeleton elasticity to play an important role in cell fate, proliferation and differentiation [186] [187]. In addition, Rodriguez *et al.* showed that dynamic arrangement of the actin network support osteogenic differentiation of MSCs [188]. Cytoskeletal tension has also been shown to regulate MSC commitment to adipogenic or osteogenic lineage [113]. Together, these studies demonstrate the importance of mechanical properties of the MSCs for stem cells-based therapy.

Our goal is to identify a subpopulation of aMSCs with similar mechanical properties to fMSCs. We therefore used a microfluidics device (fully described in § 5.2.1) to indirectly investigate "suspended state" MSC stiffness. As cell stiffness correlates with filamentous actin organization [85] and as stress fibers are absent in the suspended state, our microfluidics method measures only cortical properties [189]. Harrison et al have investigated "suspended" cells several years ago and found that cell morphology after trypsinisation depends on initial cell shape [190]. The point of using this microfluidics device was to take advantage of high throughput and ease of use with the goal of isolating therapeutically useful MSCs. Several other techniques have been explored such as fluorescence-activated cell sorting (FACS), filtration, and laser diffraction ellipsometry

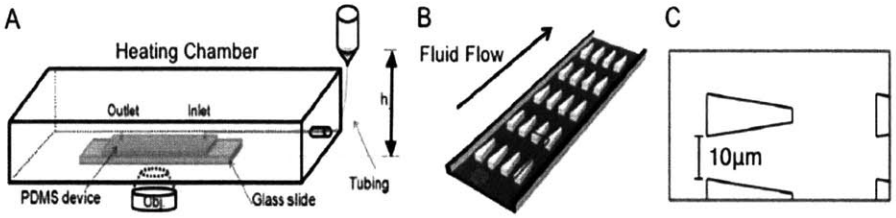
[191]. Even if these devices are good tools for measuring mechanical properties of cells, these devices required an active source of energy that could possibly affect cell viability. In addition, all these methods have more complicated set-up, lower throughput, and are more costly than our method. Hence the clear advantage to use the high throughput, simple, and relatively cheap microfluidics technique for measuring mechanical stiffness of MSCs. These experiments have been realized in collaboration with Sha Huang (MIT).

5.2.Materials and Methods

5.2.1. Design and characterization of the microfluidic device

Here we present a microfluidic device designed by Prof. Han's laboratory at MIT. This device called 'deformability cytometer' measures single cell's dynamic responses at high throughput compared to conventional methods. This high throughput enables us to measure statistically significant differences in stiffness among heterogeneous cell populations. The PDMS microfluidic device is represented in Figure 14. The design involves periodically spaced, triangle-shaped pillars. The height and minimum channel gap were chosen according to the average size of MSCs. We apply a constant pressure gradient in the device and the cells go through under low Reynolds number. The cells' dynamic deformability is extracted from their passaging velocity [191].

Figure 14: A. Schematic of the set up for live-cell measurements. B. Illustration of the device design. C. Zoomed in view of the dimension of the constriction. These three figures are adapted from Huang et al. [192].



Huang *et al.* described the fabrication techniques [192]. To summarize, a silicon wafer mold is first made, then the device is molded from the wafer using standard PDMS casting protocols and last the PDMS device is bonded to a glass slide. The experimental set up is schematically represented in Figure 14. Bow *et al.* state and explain the details of the design [191]. Bow *et al.* also demonstrated that transit time is function of stiffness of the cell [191]. We therefore in this study will use transit time of MSC, and draw conclusion on MSC stiffness.

Given the average diameter of MSCs, the present device was designed with a constriction size of 10μm. The first comment one could make is that we will not study the whole population of MSCs. Indeed, our device is not able to capture really large MSCs with this constriction size (the bigger cell we were able to measure was about 25μm).

5.2.2. Establishment of MSC culture

5.2.2.1. Adult human MSCs

The 3 "lines" of aMSCs were purchased from commercial sources: ReachBio human bone marrow mesenchymal stem cells – lot number 0090408 (referred later as RB1 – ReachBio LLC, Seattle, WA), Poietics™ human mesenchymal stem cells – lot number 7F3675 (referred later as PL2 – Lonza Walkersville Inc., Gaithersburg, MD), and Poietics™ human mesenchymal stem cells – lot number OF4266 (referred later as PL3 – Lonza Walkersville Inc., Gaithersburg, MD). All aMSCs were cultured in MesenCult® media and maintained at 37°C in a humidified atmosphere containing 5% CO_2. Medium was changed every 3 days, and cells were passaged when reaching 80% confluence.

5.2.2.2. Fetal human MSCs

fMSCs were isolated from fresh human fetal long bone as described in Chapter 3. Upon receipt of frozen cells at MIT, they were stored for 24h at -80°C, and then in liquid nitrogen. The thawing procedure is described in Appendix A. All fetal hMSCs were cultured in our homemade fetal media (90% Gibco® low-glucose Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) and 10% FBS (lot number 696409, Invitrogen, Carlsbad, CA) and maintained at 37°C in a humidified atmosphere containing 5% CO_2. Medium was changed every three days, and cells were passaged when reaching 80% of complete confluence. We called the two fMSC sources B51 and S69.

5.2.3. Culture protocols, Observation and analysis

All cell sources (the three aMSCs and the two fMSCs described above) were cultured according to protocols described in Appendix A. Experiments were performed in triplicate. The cells were seeded at 500 cells/cm^2 onto Nunc T175. 24h after thawing, the culture medium was exchanged and replaced by the same fresh medium. Every three days, the culture medium were exchanged and replaced by fresh medium. Cells were passaged onto new dishes and seeded at 500 cells/cm^2 when they reached 80% confluence.

Cells were detached from the dish using Trypsin EDTA 1X (0.05% Trypsin/0.53mM EDTA in HBSS without sodium bicarbonate calcium and magnesium – MediaTech Inc., Manassas VA – referred later as T/E) for five minutes at 37°C, and re-suspended in medium. The cells are then run through the microfluidics device describe in § 5.2.1 (The protocol to prepare the device and run the cells through is described in Appendix D). Cells were then imaged with a Hamamatsu Model C4742-80-12AG CCD camera (Hamamatsu Photonics, Japan), connected to an inverted phase contrast Olympus IX71 microscope (Olympus, Center Valley, PA). Cells 'transit times' were analyzed using the software *Origin* (OriginLab, Northampton, MA); the 'transit time' is the time necessary to the cell to go through a constriction. We measure this "transit time" on the 1st row of constrictions, as MSC's mechanical recovery after such deformation is not fully known. In our experiments, the concentration of MSCs is sufficiently low to minimize interactions between cells and guarantee that transit times are independent. As our experiment setup allowed us to measure diameter of MSCs live, we did not used the previously isolated subpopulations. We instead use the "unsorted" populations of aMSCs

and graphed our results as transit time in function of deformation index (DI)[§§§].

5.3.Results

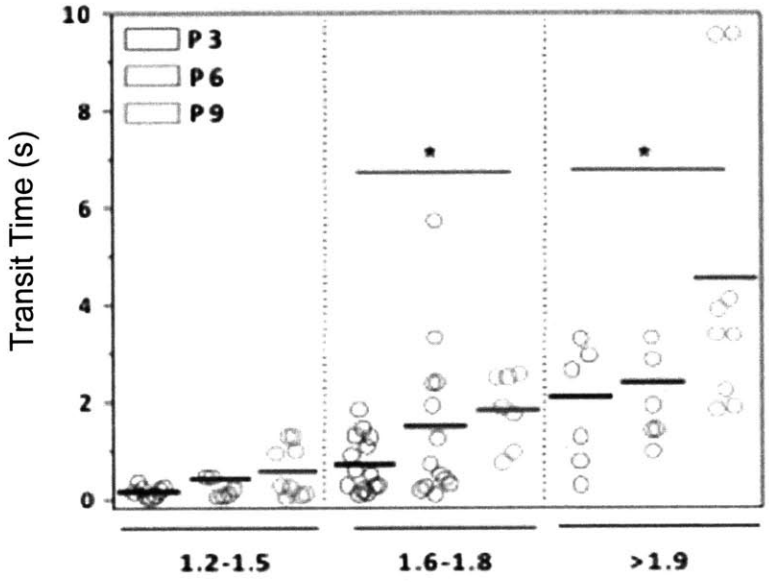
The experiments were performed under relatively constant temperature, and we therefore neglect any temperature effects on those results. We used three aMSC sources mentioned above and two fMSC sources (called later B51 and S69) at different passages. We did not use the sorted subpopulations of aMSCs in this experiment, as we were able to measure cell size with our experimental set up.

5.3.1. "Small" and "big" adult mesenchymal stem cells

We found that "small" aMSC stiffness is constant with passaging whereas "large" MSC stiffness is increasing with passaging. The data for all three aMSC sources are summarized in Figure 15.

[§§§] Deformation index is defined as the diameter of the cell over the size of the constrictions. For example, a MSC with a diameter of 15 μm going through a constriction of 10μm would have a DI of 1,5.

Figure 15: Transit time of aMSCs as a function of diameter and passage number



"Small" aMSCs' transit time is staying constant with increasing passage number. "Large" aMSCs' transit time is increasing with increasing passage number. We used a two-tailed t-test to determine if the aMSCs and fMSCs had a statistically significantly different (* $p < 0.05$) average migration speed. This results are the average from all three adult sources and two fetal sources.

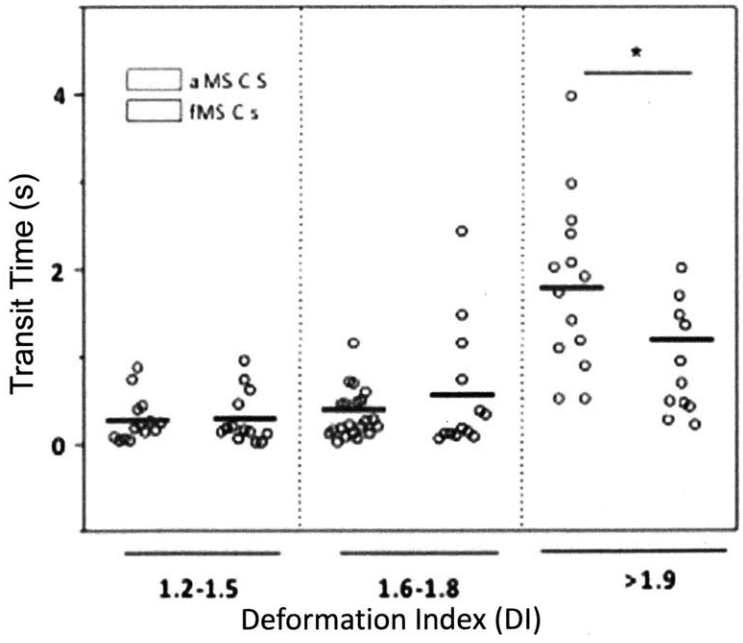
From these data, we were not able to set a clear cut-off for "small" and "large" as the threshold varied from patients to patients. We also cannot tell if the "small" aMSCs are more or less stiff than the "large" aMSCs from these data. Indeed, as all MSCs go through the same constrictions, an increase in DI means an increase in strain and therefore probably an increase in stress. To answer the question whereas if small" MSCs are more or less stiff than the "large" MSCs could be answer by performing experiments with two different gap size[****].

[****] Gap size is the size of the constrictions.

5.3.2. Mechanical properties of aMSCs compared to fMSCs

We compared transit time of aMSCs with transit time of fMSCs at three passages. We found no statistically significant differences in transit time between "small" aMSCs and fMSCs. However, we found statistically significant difference between transit time of fMSCs and transit time of "large" aMSCs. The results are shown in Figure 16. The threshold for "large" appears to be 19 μm in average for all aMSCs. However, we saw variations from patient to patient, and therefore we are not making conclusions on it in this work.

Figure 16: Transit time of aMSCs compared with transit time of fMSCs as a function of DI.



"Small" aMSCs have the same transit time as fMSCs. "Large" aMSCs have a longer transit time than fMSCs. We used a two-tailed t-test to determine if the aMSCs and fMSCs had a statistically significantly different (* $p < 0.05$) average migration speed. This results are the average from all three adult sources and two fetal sources.

5.4.Outcomes

5.4.1. Conclusions

The goal of this study was to identify a subpopulation of aMSCs with similar mechanical properties to fMSCs. The 'deformability cytometer' enabled us to quantify stiffness of MSCs relatively to each other as a function of size and passage number.

We found that stiffness of "small" aMSCs is constant with passaging whereas stiffness of "large" aMSCs is increasing with passaging. We were not able to determine what were "small" and "large" in general as the threshold varied from patient to patient.

In addition, we found no statistically significant differences in transit time between "small" aMSCs and fMSCs. However, we found statistically significant difference between transit time of fMSCs and transit time of "large" aMSCs. We estimate the threshold to be around 19 μm, but there are patient-to-patient variations. We are therefore not making conclusions on threshold in this study.

5.4.2. Possibilities for continued research

There are many appealing directions to continuing this research. First, the study could be extended to incorporate more cells. More cell sources would help us determine the threshold between "small" and "large" aMSCs. Then one could extend this work to study stiffness of "really" big and "really" small cells. Varying the gap size would be an easy way to do it. This could lead to better knowledge of the aMSC population overall.

Second and in direct connection with the work done in this chapter, it would be of great interest to determine the exact relationship between transit time and stiffness of the cell. This could put mechanical stiffness of MSCs in context with other cell stiffness, and compare results obtained with different mechanical characterization methods.

Third, one could study why "small" MSCs stiffness stays constant with passaging when "big" MSCs stiffness increases with passaging by staining actin of those MSCs and determine if increasing stiffness is coming from accumulation of actin.

Chapter 6. Conclusion

6.1. Summary of results

The first question stated in this work was whether or not "true" aMSCs exist. We hypothesized the answer was yes, and it was possible to find a subpopulation of aMSCs with similar properties to fMSCs. To verify this hypothesis, we looked at size, migration velocities, and mechanical properties of MSCs. We chose these parameters because researchers demonstrated that they were influencing proliferation and differentiation potentials. However and first of all, we tried to understand if the different culture conditions used in our laboratory could yield difference in MSCs.

Concerning culture conditions used in our laboratory, we concluded that that media and dishes used in our laboratory do not influence MSC proliferation, or at least influence proliferation in the same terms. However, the experiments showed that the plating density influences greatly MSCs proliferation. The present study did not aim to resolve general question on influence of media and/or dishes and/or plating densities over cell growth and morphology. They only suggest that culture surfaces with similar young modulus (about 2GPa) [135] and similar "enough"†††† media could be used without influencing differently MSC proliferation. Regarding our conclusions on plating density dependence of MSC proliferation, most report in the literature agrees with our findings.

The microfluidics spiral device isolated aMSCs subpopulations with different average sizes and distribution. We found that "Outlet 4" aMSCs had the closest average size and distribution to fMSCs'. The distribution of "Outlet 4" aMSCs is still a little broader and sometimes bigger in average. We also

†††† We do not try in this work to quantify this "enough".

conclude from this work that aMSCs population average size and distribution is increasing with passaging in both sorted and unsorted cases. As fMSCs population size and distribution are relatively constant over time, we believe that aMSCs-based therapy would be more efficient if the aMSCs don't need to be culture *in vitro* for long period of time.

Building on those isolated aMSCs subpopulations, we found that the smaller subpopulation, i.e. "Outlet 4" aMSCs, were the closest in migration velocity to fMSCs. Indeed, we found that the bigger the cells were, the smaller they migrated. However, fMSCs were still much faster than all subpopulations of aMSCs. So in summary, we found a subpopulation (i.e. "Outlet 4") of aMSCs that is similar in size to fMSCs, and faster than the other aMSCs' subpopulations, but still much slower than fMSCs. This could be due to an insufficient resolution to separate our aMSCs, and we could have to refine our subpopulation to see if we can identify an even faster aMSCs' sub-subpopulation. This work also aimed at determining the effect of CellTracker™ Green CMFDA on aMSCs migration velocity. We found that CellTracker™ Green CMFDA has no effect on aMSCs migration velocity and can be therefore used to facilitate MSC tracking.

At last, we found that stiffness of "small" aMSCs is constant with passaging whereas stiffness of "large" aMSCs is increasing with passaging. We were not able to determine what were "small" and "large" in general as the threshold varied from patient to patient. In addition, we found no statistically significant differences in transit time between "small" aMSCs and fMSCs. However, we found statistically significant difference between transit time of fMSCs and transit time of "large" aMSCs. We estimate the threshold to be around 19 μm, but there are patient-to-patient variations. We are therefore not making conclusions on threshold in this study. We were therefore able to show that a subpopulation of aMSCs with similar transit time to fMSCs exists even if we did not find exactly what was this

subpopulation.

6.2.Perspectives

The work presented in this document do not fully answer the question "Are there true mesenchymal stem cells or just mixed population of adipogenic, chondrogenic, and osteogenic cells?" However, this work provides insights into aMSC populations' heterogeneity.

There are many appealing directions available to continuing this research. Keeping in mind that the ultimate goal is to provide the best possible stem cell for therapy, one could start by studying the shift of the aMSCs size population. It would be interesting to know whether the shift comes from: 1- all individual cells getting bigger or, 2- only small (or large) cells becoming bigger or, 3- if the size of cells is constant, bigger cells dividing faster than smaller cells. As researchers demonstrated a link between pluripotency and size/morphology of MSCs [45], it is important to the effect of long term culturing on cell size. One could also study more functionally useful porperties such as as *in vivo* differentiation potentials, mobilization capacities, and ultimately, the therapeutic potential. Regarding our migration study, I believe that it is interesting to refine our subpopulations to find aMSCs with similar migration velocity to fMSCs. One could also reanalyze the present data and new data to quantify other important characteristics of the MSCs' migration behavior (e.g., persistence length and time, total migration distance, protrusion velocity). On a more long-term perspective, one could study *in vivo* homing of MSCs and its relationship to *in vitro* migration. This is important in our ultimate goal to isolate the "best" stem cell for therapy. Also, one could determine what is the subpopulation of aMSCS with similar transit time to fMSCs. Still related to our mechanical properties study, one could determine the exact relationship between

transit time and stiffness of the cell. This could put mechanical stiffness of MSCs in context with other cell stiffness, and compare results obtained with different mechanical characterization methods. At last, Third, one could study why "small" MSCs stiffness stays constant with passaging when "big" MSCs stiffness increases with passaging by staining actin of those MSCs and determine if increasing stiffness is coming from accumulation of actin. Also and to be sure that we are not "loosing" ourselves, we should also consider the possibility that our approach to find a "true" stem cell may be wrong and the fact could be that some cells are better at doing one thing or another. This is why researchers should keep heavily studying the subject with diverse angles.

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Appendix A: Cell culture protocols[‡‡‡‡]

Thawing, passaging, and freezing

Materials:

1. Adult mesenchymal stem cells (ReachBio, or Lonza)
2. Fetal mesenchymal stem cells (isolated in-house i [23]n Singapore by Professor Jerry Chan's laboratory)
3. Mesencult® Media composed of 90% Mesencult® Basal Media (Stem Cell Technologies #5401) and 10% Mesencult® Supplements (Stem Cell Technologies #5402) or homemade media composed of 90% Dulbecco's Modified Eagle's Medium (as basal media) (Gibco #11885) and 10% Fetal bovine serum (Gibco lot # 696409)
4. L-glutamine (200mM) (Gibco #25030)
5. Ca2+- and Mg2+-free phosphate-buffered saline (PBS, Gibco #10010)
6. Trypsin (0.05%) / EDTA (1mM) (Gibco #25300)
7. Dimethylsulfoxide (DMSO, Sigma #276855)

Methods (thawing)[§§§§]:

1. Prepare complete media: 89% basal media, 10% serum/supplements, 1% glutamine (to replenish degraded glutamine in basal media; if basal media is less than several months old, skip glutamine and replace with basal media).

[‡‡‡‡] It is assumed in all protocols that common cell biology laboratory supplies such as sterile water, 70% ethanol, and centrifuge tubes are available.

[§§§§] Adapted from Stem Cell Technologies Catalog #28453, "Technical Report: Enumeration, Expansion, and Differentiation of Human Mesenchymal Stem Cells Using Mesencult®."

2. Warm media to 37◦C.
3. Retrieve frozen cryovial of cells and thaw in 37◦C temperature controlled bath until ice is almost gone.
4. Spray vial thoroughly with 70% ethanol and transfer to sterile hood.
5. Gently transfer cells into a 15mL centrifuge tube, and add 9mL warm media drop-by-drop, swirling to mix.
6. Centrifuge at 300 g (1200 rpm) for 8 min.
7. Aspirate supernatent, resuspend pellet in approximately 1mL warm complete media, and transfer to a tissue culture polystyrene flask or dish.
8. The next day, exchange media.

*Methods (passaging)*****:

1. Check cells under a phase contrast microscope to ensure that the cells are at an adequate stage for passaging (80% confluence).
2. Aspirate media and wash the cells with 5mL PBS to remove residual serum-containing media.
3. Add 1mL Trypsin/EDTA to cover cells and incubate at 37◦C for 5–10 min.
4. Check under microscope to ensure that the cells have detached. Add 1mL complete media to neutralize the action of trypsin.
5. Collect trypsinized cells into a centrifuge tube and centrifuge the cells at 300 g (1200 rpm) for 8 min.
6. Aspirate supernatant and resuspend pelleted cells in complete media.
7. The cells can now be divided into new tissue culture polystyrene flasks or dishes. The reseeding density should be equal to the original seeding density.

***** Reagent quantities are specified to treat a T-25 (25 cm2 surface area) flask. For other culture vessels, scale quantities with surface area.

Methods (freezing):

1. Before beginning, have all reagents cold (2–8◦C) and label sterile cryovials with an indelible marker.
2. Make up 20% DMSO in FBS and filter sterilize with a 0.2 μm filter. Keep on ice.
3. Harvest cells from the tissue culture surface by using the passaging protocol described above. Centrifuge cells and resuspend in FBS at a concentration of 2×10^6 cells/mL. Place this cell suspension on ice.
4. Mix cells gently with 20% DMSO in FBS at a ratio of 1:1 (the final cell suspension will be 90% FBS and 10% DMSO). Transfer 1mL of cells in freezing media to each cryovial. The final cell concentration will be 10^6 cells per vial.
5. Place cryovials immediately into thawed 70% isopropanol freezing container ("Mr. Frosty"). Place container in −80◦C freezer overnight. (Do not let cells sit in freezing media at room temperature. Keep on ice and transfer within 5 min to the freezing container.)
6. On the next day, remove frozen vials from the freezing container and store in liquid nitrogen.

Appendix B: Microfluidics spiral channel preparation protocol

Materials†††††:

1. Mesenchymal stem cells (adult and/or fetal)
2. Mesencult® Media composed of 90% Mesencult® Basal Media (Stem Cell Technologies #5401) and 10% Mesencult® Supplements (Stem Cell Technologies #5402) OR 90% Gibco® low-glucose Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) plus 10% Gibco® selected lot FBS (lot number 696409, Invitrogen, Carlsbad, CA)
3. Bovine Serum Albumin (BSA) (Sigma- Aldrich, St Louis, MO)
4. Ca2+- and Mg2+-free phosphate-buffered saline (PBS, Gibco #10010)
5. Trypsin (0.05%) / EDTA (1mM) (Gibco #25300)
6. PhD Ultra syringe pump (Harvard Apparatus, Holliston, MA)
7. 5 mL syringes and needles
8. UV lights
9. 2 mL centrifuge vials

Methods (fabrication of the channel)‡‡‡‡‡:

First, a silicon wafer mold was made using photolithography and reactive-ion etching techniques. Then, PDMS device was casting using standard protocols. At last, the PDMS molded device was bonded to a glass slide using oxygen plasma.

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††††† Materials do not include the materials necessary for fabricating the device, but only include the materials necessary to work with the device.

‡‡‡‡‡ Devices were fabricated by Sha Huang (MIT)

Methods (preparation of the channel):

The device is kept under UV light for 30min to kill eventual germs. Then and to ensure that germs are killed, 70% ethanol is pumped through the device for 3min. At last, PBS mixed with 1% w/v Bovine Serum Albumin (BSA) (Sigma- Aldrich, St Louis, MO) is pumped through the device for 3min to coat the device walls with BSA and prevent MSC adhesion to the walls.

Methods (separation of cells by size):

MSCs are cultured using the protocol described in appendix A. Then, MSCs are detached from the culture surface, and suspended in medium at a concentration of 100,000 cells/mL to avoid interactions between cells. Those "suspended" MSCs are then pumped through the device a rate of 1.5 mL/min. A syringe pump controls the rate. An important point here is to make sure that no air bubbles are let in the device to ensure good sorting of the cells. At last, the cells are collected from the outlets in 2mL centrifuge vials.

Appendix C: MSCs staining protocol using CellTracker™ dyes§§§§§

Materials:

1. Adult mesenchymal stem cells (ReachBio)
2. Mesencult® Media composed of 90% Mesencult® Basal Media (Stem Cell Technologies #5401) and 10% Mesencult® Supplements (Stem Cell Technologies #5402)
3. L-glutamine (200mM) (Gibco #25030)
4. Ca2+- and Mg2+-free phosphate-buffered saline (PBS, Gibco #10010)
5. Trypsin (0.05%) / EDTA (1mM) (Gibco #25300)
6. Dimethylsulfoxide (DMSO, Sigma #276855)

Preparation of CellTracker® Reagent

Before opening the vial, allow the product to warm to room temperature. Dissolve the lyophilized product in high-quality DMSO to a final concentration of 10 mM. Dilute the stock solution to a final working concentration of 12.5 μM in Mesencult® Basal Medium. Avoid amine- and thiol- containing buffers. Warm the working solution to 37°C.

Methods (staining):

1. For cells in suspension, harvest cells by centrifugation and aspirate the supernatant. Resuspend the cells gently in prewarmed CellTracker™ dye

§§§§§ Adapted from http://bio.lonza.com/uploads/tx_mwaxmarketingmaterial/Lonza_ManualsProductInstructions_Instructions_-_CellTracker_Green_Fluorescent_Probe_PA-3011.pdf

working solution. Incubate cells for 45 minutes at 37°C in a humidified atmosphere containing 5% CO_2. Centrifuge the cells at 1200 rpm for 8 min. For adherent cells, when the cells have reached the desired confluence, remove the medium from the dish and add the prewarmed CellTracker™ dye working solution. Incubate the cells for 45 minutes at 37°C in a humidified atmosphere containing 5% CO_2.

2. Replace the dye working solution with fresh, prewarmed medium and incubate the cells for another 30 minutes at 37°C in a humidified atmosphere containing 5% CO_2. During this time, the chloromethyl group (and for some probes, the acetate group) of the dye undergoes modification or are secreted from the cell.

3. Attach suspended cells to glass bottom petri dish (fibronectin coated 35 mm glass bottom dishes glass bottom dish with 20 mm micro-well #0 cover glass).

4. Wash cells with PBS.

5. Add fresh medium and incubate the cells at 37°C in a humidified atmosphere containing 5% CO_2 until ready to image them.

Appendix D: Microfluidics spiral channel preparation protocol

Materials[******]:*

1. Mesenchymal stem cells (adult and/or fetal)
2. Mesencult® Media composed of 90% Mesencult® Basal Media (Stem Cell Technologies #5401) and 10% Mesencult® Supplements (Stem Cell Technologies #5402) OR 90% Gibco® low-glucose Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) plus 10% Gibco® selected lot FBS (lot number 696409, Invitrogen, Carlsbad, CA)
3. Pluronic F-108 (BASF, Mount Olive, NJ)
4. Bovine Serum Albumin (BSA) (Sigma- Aldrich, St Louis, MO)
5. Ca2+- and Mg2+-free phosphate-buffered saline (PBS, Gibco #10010)
6. Trypsin (0.05%) / EDTA (1mM) (Gibco #25300)
7. 60mL syringes

Methods (fabrication of the channel)[††††††]*:*

First, a silicon wafer mold was made using photolithography and reactive-ion etching techniques. A 5x reduction step-and-repeat projection stepper (Nikon NSR2005i9, Nikon Precision) was used for patterning. Then, PDMS device was casting using standard protocols. At last, the PDMS molded device was bonded to a glass slide using oxygen plasma.

[******] Materials do not include the materials necessary for fabricating the device, but only include the materials necessary to work with the device.

[††††††] Devices were fabricated by Sha Huang (MIT)

Methods (experimental protocol):

PBS was mixed with 0.2% w/v Pluronic F-108 (BASF, Mount Olive, NJ) and 1% w/v Bovine Serum Albumin (BSA) (Sigma- Aldrich, St Louis, MO) as a stock solution to prevent MSC adhesion to the device walls. This was the stock solution used in all of the experiments. The stock solution is pumped through the device for 30min to coat the device walls with Pluronic and BSA. In our experiments, "suspended" MSCs were diluted in the stock solution. Differences in pressure between inlet and outlet were hydrostatically by a difference in water column height. Water columns were connected to 60 mL syringes without plungers to limit surface tension effects. A Hamamatsu Model C4742-80-12AG CCD camera (Hamamatsu Photonics, Japan), connected to an inverted Olympus IX71 microscope (Olympus, Center Valley, PA) was used for imaging.