Title

The pluripotent source of mesenchymal stem cells

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Keywords

Pluripotent stem cells; embryonic stem cells; cell reprogramming; mesenchymal stem cells; mesenchymal stromal cells; differentiation; pluripotent-derived mesenchymal stem cells; iPS-derived mesenchymal stem cells; epithelial-to-mesenchymal transition; EMT.

Abbreviations

BM-MSC: Bone marrow-derived mesenchymal stem cells; EMT: Epithelial-to-mesenchymal transition; iPSC: induced pluripotent stem cells; MSC: Mesenchymal stem cells; PD-MSC: Pluripotent-derived mesenchymal stem cells; PSC: Plurpotent stem cells

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1 Abstract

Mesenchymal stem cells (MSC) have been extensively studied in the last years for the treatment of different diseases. Most of the ongoing clinical trials at present involve the use of MSC derived from adult tissues. This source may have some limitations, in particular if there is a need of extensive and repetitive dose of the cell therapy. However, there is a growing literature about the use of a new source of MSC. There is now increasing evidence about the mesenchymal differentiation of pluripotent stem cell (PSC). This cell source may overcome the previous limitations: PSC do not senecence, as MSC does. We summarize here the current knowledge of pluripotent-derived mesenchymal stem cells (PD-MSC). We present an historical perspective on the subject, and then discuss some critical questions that remain unanswered.

2 Introduction

Since the last decade we are witnessing a new frontier in therapeutic research opportunities, and the clinical translation of their results is on the verge of the bedside. The surgical and pharmacological medical models are sharing now the therapeutic spectrum with more sophisticated and complex treatments, including bioprostheses, recombinant growth factors and complex molecules, and cellular products. In this ever evolving context, mesenchymal stem cells (MSC) are being evaluated in clinical trials. In the past ten years there has been numerous clinical trials using MSC for a long list of different diseases (www.clinicaltrials.gov). However, instead of clarifying the story, there is a lot of noise regarding therapies with MSC: there are several possible cell sources, there are claims that they are useful for the treatment numerous diseases, and the proposed mechanisms of therapeutical action spanned from immunomodulation to tissue regeneration. In this complex horizon, MSC derived from pluripotent stem cells (PSC) have arrived in the last few years.

It is now well-established that MSC can be derived from pluripotent stem cells (PSC). This review intend to summarize the expanding field of pluripotent derived mesenchymal stem cells (PD-MSC). We propose to unify how these cells are called, and name them as PD-MSC, since the literature is now flooded with different names for these cells. We will go over the published research in the past years, including the many different protocols for PD-MSC development as well as the initial animal experiences aiming for clinical application. More importantly, we will discuss a few important enigmas regarding MSC-PD: what really are these cells? Are they MSC? How they correlate with adult MSC? What is their potential for clinical application? Many of these questions are not solved, but major advances has happened in the last years that promises a hot field for the near future.

3 The Stone Age: Mesenchymal versus Pluripotent Stem Cells

For many years the stem cell field was divided into the study of adult stem cells (mostly, but not only, MSC) and the study of PSC. We acknowledge that this is rather simplistic, as there are many other stem cells that do not fit into this classification, but an overwhelming part of the research was focus in these two fields.

Originally derived from the inner mass of a mouse blastocyst [1], and then from human [2], PSC are now mostly generated by cell reprogramming based on the original work from Yamanaka's group [3, 4]. The main feature of these cells is their ability to indefinitely self-renew and their ability to differentiate into any adult tissue cell, a property known as *pluripotency*. Pluripotency is readily demonstrable *in vitro* and *in vivo*. In the first instance, the cells are grown in a spheroid structure known as embryoid body, a three-dimensional cell package where the self-secreted morphogens stimulate the differentiation of the PSC into any kind of cells. *In vivo* demonstration comes from the teratoma assay, in which PSC are injected in nude mice and a few weeks later a tumor containing all sort of adult tissue can be observed. Ultimately, complementation assays provide a definitive confirmation about pluripotency [5].

Mesenchymal stem cells were suspected to exist long time ago. In the sixties, Friedenstein and coworkers discussed about the existence of

cells in the bone marrow that were unrelated to the hematopoietic lineage [6]. By implanting a bone marrow aspirate into other organ, they realized that a sort of mesenchymal cell should be present in the aspirate since a few weeks later they observed bone formation. In the nineties, Pittenger et al. published the description of a bone marrow cell population with a mesenchymal phenotype [7]. Since this description, MSC were deeply studied for their regenerative and immunomodulating properties. Although initially MSC were considered capable of transdifferentiate into other adult cells, including cardiomyocytes and neurons, soon it was realized that these cells present a limited ability to differentiate. The current evidence shows that these cells are able to differentiate into chondroblast, osteoblasts or adipocyte, and hence they are known as multipotent. The ability to immunomodulate was remarked soon after their discovery [8, 9]. This ability has been widely used in experimental and clinical trials in the past decade as the basis for treating many clinical conditions, though the definitive success of these therapies is still unproved.

Hence, up to the middle of the last decade, the growing stem cell community was divided between those working on PSC and those working on MSC. In some senses, all labs working with adults stem cells (endothelial progenitor cells, for example) could be included in the last group. Source, potency, markers, and prospects were different in these two groups. In the former, everything was seen mostly as a promise, but in the later, reality was taking the form of clinical trials.

4 The Modern Age: Mesenchymal from Pluripotent Stem Cells

Pluripotent stem cells can differentiate into any adult cells, and therefore the description of mesenchymal stem cells arising from PSC were not surprising at all. What it is surprising, as we will see, is the *easiness* with which this specific differentiation occurs. Hence, in the modern age PSC and MSC are well established different fields, but it is acknowledge that each cell can differentiate into the other.

4.1 The Context of the Embryo Development

For a better comprehension of PD-MSC it is necessary to understand the first stages of embryo development. The formation of the initial embryonic structures involves major structural changes in the pluripotent stem cells. Epiblast cells invaginate in the middle line and migrate towards the lateral processes, giving way to the formation of the mesoderm and endoderm. This is called the primary epithelial-to-mesenchymal transition (EMT). This si a critical step for embryos, although for some cells this is not the only EMT that they suffer: some structures undergoes a couple of EMT and mesenchymal-to-epithelial transition (MET) transitions before they differentiate into the adult cells [10].

During EMT, epithelial cells lose their membrane junctions and apical-basal polarity, reorganize their cytoskeleton, undergo a change in the signaling programs that define cell shape and reprogram gene expression. The epithelial cell suffer a number of distinct molecular changes such as activation of transcription factors, expression or repression of specific cell-surface proteins, reorganization and expression of cytoskeletal proteins, production of ECM-degrading enzymes, and changes in the expression of specific microRNAs.

The information about the epithelial-to-mesenchymal transition in PSC has been consistent with the richer literature describing this process in tumor cells. Several transcription factors and growth factors that orchestrate EMT during epiblast-to-mesoderm transition *in vivo* in the primitive streak during gastrulation are also involved in the initial stages of mesodermal differentiation of PSC. Multiple reports emphasize the importance of the EMT in the formation of the mesoderm-derived adult cells. Moreover, Evseenko and co-workers identified and characterized a unique population of human embryonic mesodermal progenitors (hEMP) cells, which arose from hESCs through the process of EMT [11]. These events can be easily tracked by the combined loss of the epithelial adhesion marker CD326 (EpCAM) and up-regulation of CD56 (NCAM). The analysis of hEMP cells shows the expected markers of cells undergoing

EMT.

However, the information is scarce regarding EMT in the development of PD-MSC. We analyzed the expression of some critical transcription factors that govern the EMT process such as Zeb1, Zeb2 and Snail, and found that they increase during the differentiation of PD-MSC [12]. Many cell surface markers are also down-regulated or up-regulated as expected during the EMT. Even more, there are publications about microRNAs that are critical regulators of EMT that have been found to be active in PSC, including the mir-200 family. Hence, we believe that most of the events that occur in EMT are expected to be critical for the derivation of PD-MSC.

There is no information regarding which are the critical growth factors that signal the formation of PD-MSC. Although there are a few papers that, based on the morphogens known to induce mesoderm formation [13] use these growth factors for the generation of PD-MSC, these papers only make use of this previous knowledge in order to get a population enriched in mesenchymal cells. Which one of them are critical for the differentiation of PSC into PD-MSC remains to be determined.

4.2 Derivation of Mesenchymal stem cells from Pluripotent stem cells

There has been a growing literature in the past ten years on how to develop MSC from PSC. It should not be surprising that a pluripotent cell can generate a mesenchymal stem cell, since PSC are, by definition, able to rise all kind of adult cells, including those with some features of stem cells. What it is somehow intriguing is the *easiness* of developing pluripotent derived mesenchymal stem/stromal cells, as opposed to the developing of other adult cells where complex protocols with several stages may be needed. We have recently developed a simple protocol based on the use of platelet lysate as cell media supplement, but we have observed that there is a straightforward process where PSC enters in an epithelial-to-mesenchymal transition (EMT) and eventually, and uniformly, becomes cells with all features of MSC. We have noticed that it does not matter eventually which supplements are present in the medium (i.e., platelet lysate, fetal bovine serum or defined components), but PSC cultured in the proper manner will eventually generate PD-MSC ([12] and unpublished results). It is also interesting that, provided that there are enough nutrients and survival signals in the medium, there is no evidence of a significant amount of cell death, suggesting that most PSC that were induced to differentiate will eventually do it. Although this is experimentally challenging to demonstrate, it is tempting to suggest that every PSC will become into a MSC *by default*, an event that occurs spontaneously while PSC are cultured under less stringent pluripotent conditions. Then, it is interesting that a stem cell with pluripotent and epithelial properties can directly be derived into a stem/progenitor cell with mesenchymal features, as if they were the two faces of the same coin.

After human PSC were originally described many work has been done on culture methods to keep pluripotency. Even today there are papers describing how to achieve a "higher" state of pluripotency represented by pluripotent stem cells called *naïve* or ground state pluripotent stem cell [14, 15]. Originally, PSC were co-cultured with inactivated mouse embryonic fibroblasts (iMEF). These cells were supposed to nourish PSC by secreting growth factors to the medium. Xu et al. published in 2001 explaining different substrates to culture PSC and comparing them with iMEF which represents the original methods [16]. They showed that Matrigel® and laminin, and to some extent fibronectin were good enough to maintain pluripotency when cultured with iMEF conditionated medium, but not on gelatin or plastic, which eventually will induce differentiation of the cells. However, they noticed that PSC grew under these cell culture conditions surrounded by a dense mesh of differentiated cells. These cells were observed growing from the border of the colonies. By morphology and in retrospective, this is probably the first description of cells resembling MSC outgrowing PSC. The same group later described another protocol for deriving fibroblast-like cells with the purpose of using them to support PSC culture [17]. These cells closely resemble PD-MSC derived in other

works published in the following years. The authors performed a limited characterization of the fibroblast-like cells, and found that they expressed CD44 and CD90, two known markers of MSC. These differentiated cells also lost the ability to produce teratomas, and hence, pluripotency. By those years, another paper described the methods to derive fibroblast-like cells to be used as auto-feeder cells for PSC, but the authors did not attempt to characterize these feeder cells as adult or fetal MSC [18]. In 2007, however, Ullmann et al. provided more insights into these cells that arise around the pluripotent colonies of hESC by demonstrating a mesenchymal origin of these outgrowths [19]. These authors grew the cells in Matrigel® and conditionated medium from iMEF. These cells lost the pluripotent identity and gained mesenchymal markers such as vimentin. Furthermore, these cells lost the gap junction proteins E-cadherin and connexin-43, a hallmark of the EMT in this cell population. However, the authors did not attempt to establish any connection of these cells with adult MSC.

In 2005 Barberi et al. published a paper in which it was claimed for the first time the derivation of MSC from PSC [20]. The method for obtaining these cells consists in co-culturing PSC with OP9 cells,a mesoderm embryonic cell line. After a long period (40 days), they sorted the CD73(+) cell population and found that these cells expressed several markers usually found in MSC, including CD166, CD54, CD29, CD105, CD44, and STRO-1. They found no evidence of the pluripotent markers oct-4 and nanog. These cells also differentiated into adipocytes, chondroblasts and osteoblast, as adult MSC are expected to do. Finally, they performed a genome-wide expression analysis and found a significant overlap between the genes expressed between adult bone marrow-derived MSC and those MSC derived from PSC, particularly in genes associated to the mesenchymal state. Therefore, this paper established for the first time that it was possible to derive mesenchymal stem cell from a pluripotent stem cell.

A year later Olivier et al. published a paper where they described a cumbersome method for deriving PD-MSC [21]. They called it the Raclure method after the french word *raclures*, which means scrapping. Essentially, the method consists in scrapping the differentiated cells from a standard PSC culture on iMEF. The scrapped cells were grown for 4 weeks or more and formed what they called a thick epithelial layer, although they do not provide any evidence that these cells are epithelial. These cells were then passed and after two more weeks in culture they confirmed their mesenchymal origin. Again, flow cytometry markers and multipotent differentiation of these cells showed high similitude with bone marrow-derived MSC. They also showed that these PD-MSC were able to sustain undifferentiated PSC culture as iMEF.

The list of subsequent publications on methods to derive PD-MSC is long. However, all these methods can be grouped according to a general criteria: either PSC are left to differentiate spontaneously or differentiation to MSC is directed specifically. There is a first group in which the main idea behind the method is to let them differentiate by taking out the pluripotent signals in the medium. For example, Trivedi et al. grew PSC onto Matrigel®-coated plates with iMEF conditionated-media but spacing the medium changes from 3 to 5 days [22]. This step induced the appearance of cells with a MSC morphology around the PSC colonies. Although this efficiently differentiated cells into a MSC-like phenotype, they still had to manually dissect and extract the undifferentiated colonies, a step that had to be repeated several times. These authors also showed for the first time that these cells could immunomodulate and reduce the proliferation of lymphocyte upon stimulation. Another publication also mechanically separated the mesenchymal cells appearing from embryoid bodies attached after 10 days of differentiation [23]. Then, coming from undifferentiated PSC in colonies or differentiated PSC in the form of embryoid bodies did not seem to make any difference. Therefore, these initial approaches to PD-MSC derivation were generated by the spontaneous appearance of mesenchymal cells coming from undifferentiated or pre-differentiated cells. In these first papers on PD-MSC, no specific stimulus was given to the PSC in order to generate mesenchymal stem/stromal cells, and then these cells can be seen as by-stander of the spontaneous differentiation of PSC, which may

happen when the culture conditions are not sufficient to maintain the pluripotent state. Given adverse conditions to sustain pluripotency, PSC enters in EMT and acquires a mesenchymal phenotype. These previous methods to derive PD-SC were effective, but still required some specific manipulation and the use of FBS to induce differentiation.

A second group of papers were published with more specific methods. Stavropoulos et al. showed that it was possible to obtain PD-MSC after culturing hESC for 21 days in ITS (insulin-transferrin-selenite) and then for 1 more week in FBS [24]. After this period they sorted CD73(+) cells, which presented all features of MSC. Karlsson et al. also showed that it was possible to differentiate PSC into PD-MSC by several passages with trypsin and using a medium supplemented with FBS and bFGF [25]. In the same year Boyd et al. showed a similar result by growing PSC in endothelial medium for a few weeks [26]. They observed that in the colonies of PSC appeared sheets of epithelial cells. After 20 to 30 days, they passed the cells that, in turn, underwent epithelial-mesenchymal transition with the appearance of PD-MSC. Lee et al. also published their methods to derive PD-MSC based on the outgrowth from embryoid bodies also in an endothelial cell culture medium [27].

A third group with protocols which use specific inhibitors and/or growth factors were finally published. All previous protocols were performed with medium supplemented with fetal bovine serum, which provides multiple growth factors with non-specific signals to the cells. Instead of inducing a non-specific differentiation signals, these protocols include specific signals by incubating the cells with known morphogens that drives the PSC to mesoderm formation. For example, Mahmood et al. used the TGF-β inhibitor SB-431542 during the differentiation of pluripotent stem cells in embryoid body structures [28]. Another paper by Sanchez et al. used a similar strategy by using the same TGF-β inhibitor, but this time in two dimensional growing cells [29]. The fact that these authors used an inhibitor of the TGF-β family is interesting since the inhibition of TGF-β have been implicated in the maintenance of the undifferentiated state of the pluripotent stem cells [30, 31]. Moreover, in a complex protocol Kimbrel et al. develop PD-MSC by growing the cells first in embryoid bodies and then in 2D conditions, incubating them with bFGF, Vascular Endothelial Growth Factor (VEGF), Bone Morphogenetic Protein 4 (BMP4), and thrombopoietin [13]. Wu et al. used the ROCK inhibitor Y-27632 and the neural stem cell supplements B27 and N2, getting a defined medium for the differentiation of PSC into MSC [32]. Again, these protocols were shown to be effective in differentiating PSC into PD-MSC, either by finding expression of the usual mesenchymal surface markers, multipotentiality or by immmunomodulation. Even though these protocols can be seen as easier and clinically compatible, they are usually much more expensive.

We have recently published our experience with a new protocol to derive PD-MSC [12]. We found that these cells can be easily grown from hESC or iPSC when they are culture for at least 3 weeks in a medium supplemented with human platelet lysate. We analyzed the temporal pattern of the differentiation process, either by gene expression analysis or by flow cytometry and established that 21 days is approximately the time needed to fully differentiate. We compared the expression of a large list of cell surface markers and performed functional analysis on these cells and found a phenotype compatible with MSC. Moreover, we compared the expression profile of PD-MSC with umbilical cord-derived MSC and fibroblasts, and found minor differences between them. We also performed the same protocol but using FBS instead of platelet lysate, or defined supplements such as a combination of growth factors and small molecules (bFGF/BMP4/Lithium chloride or the TGF-beta inhibitor SB431542) and found that they also were able to differentiate PSC into PD-MSC with no major differences (unpublished results). A similar finding was done by Diederichs and Tuan [33]. They analyzed PD-MSC derived by four different protocols and found no majors differences in terms of MSC differentiation. However, they report some heterogeneity in the final MSC population. Therefore, these findings stressed out that there are many ways to obtain PD-MSC, and although we performed some comparison between different supplements, there is no formal comparison between all these

published protocols. To what extent the obtained PD-MSC by these different protocols are equivalent, or if all of them correspond to a similar mesenchymal cell type, is unknown, but they seem to share all the classical features of a mesenchymal phenotype.

In summary, there is a large list of differentiation protocols that would eventually produce PD-MSC from PSC. These protocols can be grouped into three general concepts. First, there are protocols that are mainly based on the isolation of differentiated cells that arise around PSC colonies, usually after a change in the usual culture techniques that keep the cells in a pluripotent state. These protocols mechanically collect the cells with a change in the morphology, and then subculture them until the typical MSC morphology appear. A second group of protocols include those that involve a more active induction of the mesenchymal differentiation. In these cases, the protocols usually introduce defined cell culture mediums and eventually separate the differentiated cells by surface markers. Finally, more specifics protocols have been published where PSC are directed to differentiate by means of specific growth factors and pathway inhibitors. These protocols are based in specific signals that are known to induce mesoderm formation. Even though these general differences can be observed, there are similarities that deserve to be mentioned. First, it usually takes several weeks to get fully differentiated PD-MSC. Second, all these protocols result in an homogeneous mesenchymal cell population, without contamination with cells with other cellular phenotypes. Third, differentiation is complete, and no remnants of undifferentiated cells are found. Finally, all these protocols are described as robust and consistent in their results.

4.3 Pluripotent-derived Mesenchymal stem/stromal cells characterization

A critical question on the subject of PD-MSC is about their definition and characterization. The question if a PD-MSC is a true MSC has been challenged, but there are still some controversies about these facts. Regarding the characteristics that define a MSC, a few years ago an expert committee gave their recommendations [34]. But these criteria can be considered as an approximation to (and a reduction of) a complex subject, and hence many other criteria can be properly used to assert that a cell is a MSC. The application of these criteria to PD-MSC was immediate, and it can be observed that PD-MSC are positive for all these criteria with minor differences. However, we think that it is also important to demonstrate that the PD-MSC population is originated from undifferentiated cells that underwent EMT with the appearance of all mesenchymal features. This finding supports the notion that the cells are indeed a mesenchymal derivation. Finally, the demonstration that there is no persistent feature of pluripotency is also important.

The tissue of MSC source is widely used to help define MSC, but even tissue-specific populations seem to contain subsets of MSC. So, how equivalent are PD-MSC to MSC from other sources? This question is hard to answer and it will depend on the criteria of each reader to say how similar is a PD-MSC to a MSC. It cannot be denied, however, that in general PD-MSC presents many of the structural and functional features of a MSC. We attempted to clarify this issue by comparing PD-MSC with umbilical cord-derived MSC (a cell population developmentally closer to pluripotent cells than adult MSC) [12], as well as other authors have used mesenchymal cell lines derived from embryo tissues [21], and we found no major differences between mesenchymal cell populations.

Another approach to answer this question has been the use of genome-wide expression analysis [20, 21, 35–37]. As expected, these papers describe a significant overlap in gene expression between PD-MSC and bone marrow-derived MSC. These genes include many that are well-recognized markers of mesenchymal cells. Moreover, the differences can be obviously expected considering the source that may imprint the origin of the cells in a niche-related way. One interesting finding is that mesenchymal cells inherit some important features from their origin. For example, cells derived from PSC has a shorter doubling time and longer telomeres than MSC derived from the bone marrow, a feature that resembles the characteristics from

the pluripotent stage [38]. These finding give PD-MSC the property of a fast expansion after the differentiation, making them attractive to use for experiments and preclinical trials. Recently Billings et al. compared PD-MSC with BM-MSC using genome-wide and proteomic analysis [37]. This extensive analysis of both MSC strongly support that PD-MSC are indeed a mesenchymal cell.

Surface markers have been a standard for the identification of the MSC population. There is a vast array of markers that have been found in the surface of these cells, and there is no unique marker or pattern that distinguishes MSC from other cells. The classical pattern of CD90(+)/ CD73(+)/CD105(+) is also present in PD-MSC, although we found that CD90 is also highly expressed in undifferentiated cells [12]. Most MSC markers described in the literature have also been found to be expressed in PD-MSC. An exception could be the mesenchymal marker Stro-1; we and others found that PD-MSC are negative for this marker [32]. Interestingly, fibroblasts also expressed all these markers, a finding that supports the few differences that can be found when MSC are compared to fibroblasts [39]. Moreover, there is also a clear change in the pattern of marker expression compared to PSC. As explained, the development of PD-MSC involves an EMT. Therefore, there is a switch in the surface marker expression where PSC lost their pluripotent markers (SSEA-4, Tra-1-60, CD-326, E-cadherin, etc.) and gain the mesenchymal ones. We also found a change in the expression of some integrins. PSC are negative for CD49a, CD49d and CD51/61, but strongly expressed them after MSC differentiation.

Mesenchymal stem/stromal cells also present the multipotent ability by which they can differentiate into adipocyte, chondrocytes and osteoblasts. Again, multipotency has also been widely used to demonstrate the MSC phenotype of the PD-MSC cells. Most publications readily demonstrate this ability in these cells, although it has been reported that these cells are less multipotent when compared to the adult bone marrow-derived MSC [33, 40].

Finally, another characteristic of MSC is their immunomodulatory ability. Many papers have also reproduced this ability in PD-MSC, and even more, some papers suggested that this feature is even more potent in PD-MSC [41], though this finding failled to be confirmed in another *in vitro* study [42]. In any case, PD-MSC reproduce MSC strong inhibition of activated lymphocyte proliferation [12, 13, 22, 29, 41]. Finally, studies regarding the mechanisms by which PD-MSC immunomodulate are scarse [43–45], but it is supposed that they are probably similar to those found in MSC from both adult and neonatal tissues.

5 The Future Age: Experimental therapies with PD-MSC

MSC are a promising source of cells for therapy. Currently there are many clinical trials evaluating the effects of MSC in a variety of diseases, including osteoarthritis, wound healing, degenerative disease, and autoimmune disorders (U.S. National Institutes of Health; www.clinicaltrials.gov (2016)). One of the proposed advantages of MSC for cell therapy is that these cells are able to evade immune detection. Although the exact mechanism of this property is still not clear, now we know that MSC do not express co-stimulatory molecules such as CD80 or CD40, they may express HLA-G and a non-canonical MHC class I molecule [46] and a serine protease inhibitor of the immune response [47]. All these mechanisms may contribute to their immunopriviliged status.

Although MSC may be readily isolated from several different adult tissues and are being incorporated as an alternative cell source in regenerative therapy, from a pharmaceutical point of view there are still some issues left to solve in order to make MSC readily available. First, MSC looses their multipotency and immunomodulatory properties when cultured for long periods of time [48, 49]. This poses several concerns over the possibilities of scaling up MSC culture to meet clinical demands. On the other hand, the isolation of adult MSC from different sources or different donors gives to the cell preparation a degree of heterogeneity, which is a problem for cGMP validation. In this regard, PD-MSC might be a clever solution for medical industry. Since PSC can self-renew,

the source for MSC derivation can be validated and reproducibly differentiated to produce large quantities of young, fresh MSC at low passages. Recently, a publication supports this fact since it has been shown epigenetic changes compatible with a reversion of cellular aging [50]. The research with PD-MSC developed so far falls well behind to the adult or neonatal MSC, but recently an Australian-based company has announced that they will conduct a phase I clinical study with PD-MSC in graft-versus-host disease (Cynata Therapeutics).

Though there are no current clinical trials involving PD-MSC, but a wave of animal studies using these cells have occur in the past few years (Table 1). For example, in 2014 Kimbrel et al. showed that PD-MSCs have therapeutic efficacy in two different autoimmune disorder models, including a marked increase in survival of lupus-prone mice and a reduction of symptoms in an autoimmune model of uveitis [13]. Contemporaneously, Wang and co-workers postulated that PD-MSC have significant better performance than bone marrow MSC in treating and Experimental autoimmune encephalomyelitis (EAE) model of Multiple Sclerosis [41]. In fact, bone marrow MSC were ineffective in this model. Many other animal studies are coming out, replicating the findings of MSC from other sources. These works are showing that PD-MSC are effective and safe as immune modulators in animal models of inflammation and autoimmunity [51–55].

Finally, exosomes collected from PD-MSC were shown to enhance cutaneous wound healing in rats by promoting collagen synthesis and angiogenesis [56]. There are a few publications with PD-MSC that showed in *in vivo* animal models a similar clinical efficacy than adult MSC [57–59]. The field of MSC-derived exosomes is rapidly growing [60], and it can be expected in the near future that PD-MSC derived exosomes will also be clinically investigated.

6 Unanswered Questions with PD-MSC

The field of PD-MSC is expanding every year. There are now many publications showing that these cells truly resemble MSC, and that they behave as adult MSC. However, several questions still remains about PD-MSC. Research in the future will probably address this topics.

- *Why is is relatively simple to differentiate PSC into MSC?* Although this question sounds vague, the fact that many different protocols yields similar PD-MSC support the concept that it is relatively easy and straightforward to obtain these cells. It has been reported that the downregulation of the main pluripotent transcription factors in PSC induces the appearence of a trophoblastic phenotype [61, 62]. Hence, are PD-MSC a sort of extra-embryonic mesenchymal stem cell?

- *What is the main driving force for differentiation?* If many different signals induces the differentiation of PSC into MSC, with no major apparent differences in the final fate, which is the major signal or signals that lead to the straightforward differentiation, if there is any? The understanding about the signals that induced mesoderm differentiation in PSC are well described. However, is there any specific signaling for PD-MSC differentiation? Alternatively, the differentiation towards PD-MSC could be a default process under still unknown circumstances?

- *How differentiation protocols compare to each other?* There are many ways to induce the differentiation of PD-MSC, but, are all these cells the same? Heterogeneity is well described in MSC isolated from adult tissue. However, there is no information if this heterogeneity exist in PD-MSC, or if they are an uniform cell population.

- *Are they safe?* There is a vast literature showing that MSC from other sources are safe. Now, can we consider that PD-MSC are safe? What do we need to be confident that these cells are stable, and that they will not produce a tumor? Considering their origin from PSC, this concern needs further research in the future.

7 Concluding remarks and future directions

The ability of PSC to differentiate into MSC have been explored in the past ten years. There are multiple protocols that are able to produce cells with all the features that characterize a MSC. We believe that there are some reasons why these cells may become key players in the field of regenerative medicine and MSC research in the near future. First, they may be easier to produce and have a higher proliferation rate, with less senescence. Second, once iPSC cells are obtained from a patient, there is potentially an unlimited source of MSC to work with. iPSC can be considered as inmortal, and hence they can be seen eventually as an off-the-shelf bone marrow-like tissue to produce MSC. At last, we believe that exosomes from PD-MSC may well combine a successful scheme for a therapeutic product, where iPSC generates an unlimited amount of MSC and they eventually produce large quantities of exosomes in an easy and cGMP compatible way.

Some hurdles, however, should finally be mentioned. The research done so far with PD-MSC shows a promising future, but there are still critical unanswered questions. How are these cells formed, and which are the key signals necessary for an effective differentiation? Are all cells generated and all protocols producing the same type of PD-MSC? Is there any variability regarding the genetic background of the iPSC cell? Do any previous disease, such as diabetes, affect the source and outcome of PD-MSC production? The derivation of MSC from PSC is well described, but giving its initial steps in terms of demonstrating their research and clinical utility, we foresee an active and exciting incoming years in this field.

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Table 1: Animal models using PD-MSC.

Reference	Animal Model	Results
Hwang et al., 2008 [23]	Cartilage-excision model in mouse	PD-MSC differentiate into chondroblast and were able to repair the defect in mouse.
Arpornmaeklong et al., 2009 [63]	Calvarial bone resection model in mice	PD-MSC effectively regenerate bone in this model.
Sánchez et al., 2011 [29]	Colitis-induced mouse model	Significant reduction in bowel inflammation, with less neutrophil invasion.
Zhang et al., 2012 [64]	Monocrotaline-induced pulmonary hypertension mice model	PD-MSC were significantly better than BM-MSC in reducing pulmonary pressures. PD-MSC integrated to the pulmonary vasculature and differentiated into endothelial cells.
Himeno et al., 2013 [65]	Streptozotocin-diabetic mouse model	PD-MSC were injected in the muscle. Neurophysilogical test significantly improved.
Li et al., 2013 [66]	Mouse model of hematopoietic CD34(+) stem cell transplantation	PD-MSC supported CD34(+) cell grafting in the bone-marrow. The authors claimed that PD-MSC did not reduce *in vitro* lymphocyte proliferation.
Kimbrel et al., 2014 [13]	Lupus-prone mice with nephritis and an uveitis mouse model	Increased survival in lupus-prone mice; significantly reduction in inflammatory cell infiltration in eyes tissue in the uveitis model.
Wang et al., 2014 [41]	Mouse experimental autoimmune encephalitis	PD-MSC were significantly better than BM-MSC to reduce signs of brain inflammation. In fact, BM-MSC has no positive effects, probably due to IL-6 secretion by these cells.
Miao et al., 2014 [67]	Myocardial infarction in mice	Significant reduction in adverse left ventricle remodeling, with improvement in angiogenesis.
Gonzalo-Gil et al., 2015 [44]	Collagen-induced arthritis in mice	PD.MSC significantly reduced joint inflammation, increased regulatory T-cells. the effect was mediated by IDO secretion by host cells.
Cheng et al., 2015[45]	Islet transplantation in a streptozocin-induced diabetic mouse model	The addition of PD-MSC significantly improves graft survival. There was less immune cell infiltration and increased number of regulatory T-cell.
Hao et al., 2015 [51]	Acute pulmonary injury	Improvement in pulmonary function after sepsis induced damage by E. coli. PD-MSC outperformed BM-MSC.
Hu et al., 2015 [58]	Mouse model of hind-limb ischemia	Exosomes collected from PD-MSC, significantly improved limb perfusion after 30 days.
Zhang et al.; 2015 [53]	Doxorubicyn-induced cardiomyopathy	Significant improvement of ventricular function with PD-MSC, event better than BM-MSC. The effect was mediated by the secretion of MIF and GDF-15 by the PD-MSC.
Hajizadeh-Saffar et al., 2015 [52]	Diabetic nude mouse model	Co-injection of modified PD-MSC with VEGF expression with pancreatic islets. Increased survival and performance of the grafts.
Ferrer et al.; 2016 [54]	Canine model of anal fistula	Good results at three months, although they found several relapses at 6 months. Cyclosporine,was use for tolerance induction.