IN SEARCH OF *IN VIVO* MSC

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IN SEARCH OF *IN VIVO* MSC

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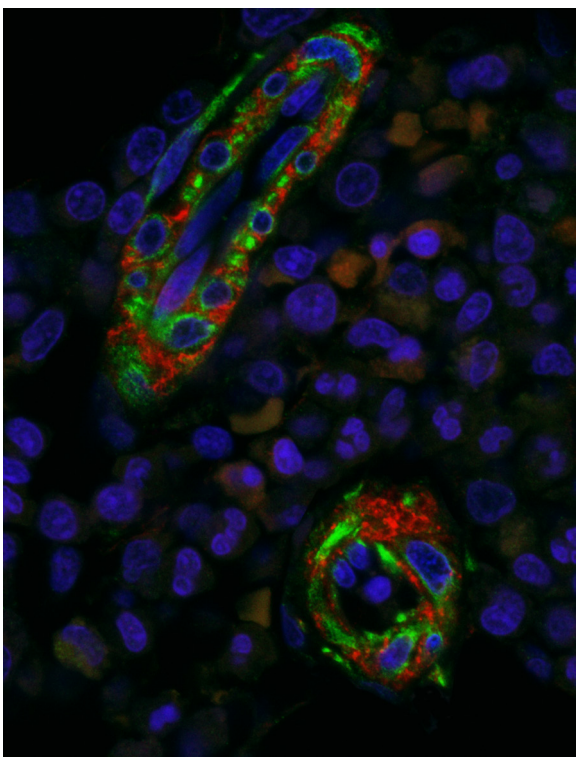

Although MSCs have been largely studied for their interesting applications in clinical trials, several aspects of MSC biology are still unclear. The heterogeneity and morpho-functional variability of MSC cell preparations could be expression of the effects of culture conditions. Culture determinants can indeed select, or simply promote, particular subpopulations of MSC-like cells. Recently, perivascular localization of MSC precursors may explain their presence in a wide range of tissues and organs. In particular, in bone marrow pericytes has been identified as the major source of MSCs and the positivity to nestin has been decribed in correlation with the establishment of the hemopoietic niche. In this figure, immunofluorescence on bone marrow slice for CD146 (red) and nestin (green) reveals the perivascular localization of the cells that has been considered the *in vivo* progenitor of bone marrow MSCs.

The concept of multipotent mesenchymal stromal cells (MSCs) arose from the work of A. J. Friedenstein and coworkers in which the authors observed that culturing human bone marrow (BM) cell suspensions, in plastic dishes, lead to isolation of proliferating adhered colonies of fribroblastoid cells able to differentiate into chondrocytes or osteoblasts, in vitro and *in vivo*. Authors firstly described these cells as colony forming units of fibroblastoid cells (CFU-Fs) referring to their ability to form large colonies on plastic surfaces.

The acronymous "MSC" became popular after the work of A. I. Caplan et al in 1991 where the authors proposed that in adult BM, a population of stem cells could differentiate into different tissues originated from the mesodermal layer, during embryonic development. They termed these cells as "mesenchymal stem cells" (MSCs). Later, the multilineage differentiation capability of MSCs was then definitively demonstrated, these cells shown a stable phenotype expressing novel markers as CD105, CD73 and CD90 and could be expanded retaining the ability to differentiate, in vitro, into vary mesodermal tissues. Some investigators described these latest findings as the definitive characterization of the culture expanded CFU-F population originally described by Friedenstein group, but the identity of the putative *in vivo* MSC remain enigmatic.

Emerging interest in identifying the MSCs *in vivo* counterpart in order to indicate feasible prospective isolation methods lead to increasing number of ex vivo isolating immunological procedures. Nonetheless, any effort failed to describe a definitive and widely accepted protocol, and significantly contributed to the ongoing confusion in the description of the *in vivo* MSC identity. Meanly, the inconclusive data about isolation of the putative MSC progenitor could be ascribed to the assumption that any marker expressed on culture-expanded MSCs was also likely to be present *in vivo*. Consequently, independent laboratories have begun to use different markers of cultured MSCs to search for MSCs in the source tissue. This has resulted in the perception that these *in vivo* progenitors were highly heterogeneous cell population and that the different protocols applied could lead to the isolation of distinct sub-populations showing increased CFU-F frequencies.

This issue is organized in two sections. In the first section, there are collected articles regarding the effects of culture determinats on the heterogeneity of MSC preparations, and how to interpret data from culture expanded cells. The second section presents contributes regarding the impact of MSCs and their *in vivo* counterpart on health and disease.

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EDITORIAL
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Editorial: In Search of *In vivo* MSC

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Keywords: *in vivo* MSC, MSCs, mesenchymal stem cells, multipotent stem cells, multipotent cell differentiation, bone marrow transplantation, adult stem cells

Editorial on the Research Topic

In Search of *In vivo* MSC

The concept of multipotent mesenchymal stromal cells (MSCs) arose from the work of Friedenstein et al., in which the authors observed that culturing human bone marrow (hBM) cell suspensions, lead to isolation of proliferating adhered colonies of fribroblastoid cells able to differentiate into chondrocytes or osteoblasts, *in vitro* (Friedenstein et al., 1968), and *in vivo* (Friedenstein et al., 1974). Emerging interest in identifying the MSCs *in vivo* counterpart lead to increasing number *of ex vivo* isolating procedures. Nonetheless, any effort failed to describe a definitive accepted protocol, and significantly contributed to the ongoing confusion in the *in vivo* MSC identity (Trombi et al., 2009; Cordeiro-Spinetti et al., 2014; Montali et al., 2016). This resulted in the perception that these *in vivo* progenitors were highly heterogeneous cell population.

Due to this uncertainty, after several decades from its first description the colony-forming units assay remain the elective indirect method to quantify mesenchymal progenitors in a cell suspension. Nonetheless, it is known that colonies are highly heterogeneous and arise from a single progenitor with different level of "stemness" or "commitment" (Muraglia et al., 2000; Russell et al., 2010), which may correlate with colony size and cell distribution (Kuznetsov et al., 2009). In this issue, Eric Cordeiro-Spinetti and colleagues discuss how variability in FBS could affect CFU-F size, frequency and morphology. Authors suggest a "*Selective Growth Hypothesis*" where distinct mesenchymal progenitors could be differentially induced to proliferate or to remain quiescent depending on serum concentration of different growth factors (Cordeiro-Spinetti et al., 2014).

We had also proposed the "*Selective Growth Hypothesis*" explaining the emergence of a new stromal progenitor in primary cultures of hBM cells (Trombi et al., 2009). These cells, recently renamed "Mesangiongenic Progenitor Cells" (MPCs) retaining both mesengenic and angiogenic potential (Montali et al., 2016). MPCs could be easily obtained culturing hBM cells in minimal essential medium supplemented with pooled human AB type sera (PhABS) in place of FBS. However, the efficient MPC isolation had always been affected by a great lot-to-lot variability. Here, Montali et al. demonstrated that this variability is in close correlation with the concentration of few specific growth factors, supporting the hypothesis propose by Cordeiro-Spinetti's group.

Most of the controversies still debated on MSC differentiating/supporting potential, could be ascribed to the heterogeneity of MSC culture products and the variability related to their fabrication. Unfortunately, there are still no phenotypical and molecular markers that could be efficiently applied evaluating the potential of a MSC primary culture. Here Wilson et al. investigated the possibility to apply glycan profiling to predict the cell differentiating potential. In

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their interesting new approach, Authors compare *N*-Glycomes from different MSC clones showing distinct osteogenic activity, *in vitro*.

In addition, one the most debated controversy in MSC biology is represented by the genuine angiogenic potential of these cells. In this issue, Iacopo Petrini and I try to answer to the question: "Are MSC angiogenic cells?" hypothesizing that the presence of undetected MPCs, could be responsible for the angiogenic potential of MSC cultures reported by some Authors but not yet definitely demonstrated (Pacini and Petrini).

Here above, the "*Selective Growth Hypothesis*" has been discussed in correlation to the concentration of particular growth factors. Nonetheless, many others culture parameters could deterministically and stochastically varying during the culture time as cell density, pH, temperature, nutrient impoverishment, medium evaporation and oxygen tension (Pacini, 2014), resulting in cell stressing conditions. Under those conditions some particularly stress resistant sub-populations could be positively selected. Ciavarella et al. explored this hypothesis, applying extremely adverse conditions to cultures of vascular wall-derived MSC and reporting interesting results on cell survival. Moreover, Antonini et al. suggest that also nanotopography of the culture surface should be taken in account as further culture determinant Antonini et al. These results corroborate the hypothesis that an untreatable number of external factors contributing to the heterogeneity of *in vitro* isolated/expanded populations.

BM still represents the most extensively studied source of MSCs and the hypothesis that these cells could migrate from BM to other injured organs triggering tissue regeneration has gaining evidence. Kimura et al. demonstrate the recruitment of BM-derived MSC progenitors in periodontal tissue defects produced in a chimeric mouse model. Moreover, it has been demonstrated that these cells could be recruited also by tumor tissues in respond to inflammatory molecules and MSCs have been considered contributing to cancer progression. In this topic, Kudo-Saito summarizes current knowledge about the role of MSCs in tumor aggravation.

BM-derived cells could also be applied to allogeneic BM transplantation (BMT) where hemopoietic stem cells (HSCs) and MSCs could be systemically administered, restoring normal hemopoiesis thank to their "*homing*" ability and BMT also represents a therapeutic approach to autoimmune diseases. However, some reports demonstrated that stroma cells could be trapped in the liver when are systemically infused. To overcome this obstacle, Professor Ikehara's group defined the methodology to directly inject BM into the bone cavity, demonstrating that this methods results more effective in allogenic BMT. This Research Topic hosts an interesting review article about the application of *intra-bone marrow-bone marrow transplantation* (IBM-BMT) in the treatment of rheumatoid arthritis and malignant tumors (Li et al.).

Expanded and *ex vivo* isolated MSCs from different tissues of origin has been under extensive investigations also for the treatment of heart diseases, which have a great impact on public health. Professor Paolo Madeddu, in collaboration with Paolo Caputo and Elisa Avolio, here presented a comprehensive review on stem cell therapies for congenital heart diseases (Avolio et al.).

Although MSCs might be considered the most intensely studied adult multipotent cells, comparison of existing pre-clinical and clinical data reveals a significant level of uncertainty. Most of the reduced predictability of pre-clinical studies could be ascribed to the uncertainty about the genuine MSC *ex vivo* ancestors. The identification of these cells could be affected by an untreatable number of variables related to the donors, the tissue of origins and cell manipulations.

Thus, the application of highly purified cell populations, finely characterized and unequivocally defined by specific manipulation procedures is of primary importance. All the contributions collected in this Research Topic strongly suggest avoiding the generic definition of MSC to characterize the various multipotent cell populations object of study.

AUTHOR CONTRIBUTIONS

SP: Revised the literature and drafted the article, MP: Revised the literature and final approve.

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Human bone marrow mesenchymal progenitors: perspectives on an optimized *in vitro* manipulation

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When it comes to regenerative medicine, mesenchymal stem cells (MSCs) are considered one of the most promising cell types for use in many cell therapies and bioengineering protocols. The International Society of Cellular Therapy recommended minimal criteria for defining multipotential MSC is based on adhesion and multipotency *in vitro*, and the presence or absence of select surface markers. Though these criteria help minimize discrepancies and allow some comparisons of data generated in different laboratories, the conditions in which cells are isolated and expanded are often not considered. Herein, we propose and recommend a few procedures to be followed to facilitate the establishment of quality control standards when working with mesenchymal progenitors isolation and expansion. Following these procedures, the classic Colony-Forming Unit-Fibroblast (CFU-f) assay is revisited and three major topics are considered to define conditions and to assist on protocol optimization and data interpretation. We envision that the creation of a guideline will help in the identification and isolation of long-term stem cells and short-term progenitors to better explore their regenerative potential for multiple therapeutic purposes.

Keywords: bone marrow, mesenchymal stem cell, colony, *in vitro* expansion, mutipotent progenitor

INTRODUCTION

To minimize discrepancies and inconsistencies, and allow comparison of data generated in different laboratories, members of the International Society of Cellular Therapy (ISCT) (Horwitz et al., 2005) have recommended minimal criteria for defining multipotential mesenchymal stem cells (MSCs). By ISCT criteria, MSCs must adhere and grow on a substrate *in vitro* and give rise to osteoblasts, chondrocytes, adipocytes, and hematopoiesis-supporting reticular stroma when cultured under proper differentiation conditions. MSCs must also express CD73, CD90, and CD105, but not express hematopoietic cells and endothelial cells markers (Barry et al., 1999, 2001; Jones et al., 2002; Horwitz et al., 2005; Dominici et al., 2006; Sarugaser et al., 2009). Further investigation unveiled a few other surface markers, among which CD146 has been demonstrated to be consistently expressed by all MSCs and progenitors (Bianco et al., 1988; Shih, 1999; Dennis et al., 2002; Tuli et al., 2003; Zannettino et al., 2003; Sacchetti et al., 2007).

Growing evidence indicates an intimate relationship between MSCs and those cells identified as pericytes, since these two populations demonstrate similar behavior and potential *in vitro* and *in vivo* (Shi and Gronthos, 2003; Sacchetti et al., 2007; Taichman et al., 2010; Péault, 2012). Pericytes are perivascular cells which reside on the abluminal side of sinusoids and are known to express the proteoglycan NG2, alpha smooth muscle actin (αSMA), and Platelet Derived Growth Factor Receptor (PDGFR) (Andreeva et al., 1998; Crisan et al., 2008a, 2009; Maier et al., 2010). Similarities to pericytes led to the concept that all tissues in the body harbor their own population of mesenchymal-*like* stem cells. Of note, it is important to stress that these cells are influenced by the niche they occupy *in vivo*, making them similar to each other, but with a few distinct characteristics and differentiation bias. Mesenchymal-*like* stem cells and progenitors have been isolated from several tissues, but adipose tissue and bone marrow are usually indicated as most promising sources of these cells by those working in the cell therapy and bioengineering fields (Da Silva Meirelles et al., 2006; Crisan et al., 2008b; Corselli et al., 2011). Yet to date, no specific or combination of markers can be used to distinguish multipotential MSCs from committed progenitors. A differentiation cascade, similar to the hematopoietic system, has not yet been assembled and confirmed.

Friedenstein and coworkers (Friedenstein et al., 1974a,b; Friedenstein, 1976; Owen and Friedenstein, 1988) were the first to describe the existence of a second category of progenitors residing in the marrow cavity, and named them stromal progenitor cells. His cues came with an *in vivo* assay, in which bone marrow cells were loaded into chambers and implanted subcutaneously in rats (Friedenstein et al., 1966, 1974b). After several weeks of implantation, bone-*like* mineralized nodules and cuboidal osteoblasts were observed inside the chambers in the new-formed tissue. The chamber's pores were too small and prevented cells from migrating into or out of the chambers, supporting the concept

that the new bony tissue formed inside the chambers was exclusively generated from donor cells rather than recipient cells. Additional studies identified this activity belonging to the non-hematopoietic stromal fraction. *In vitro*, they showed that when bone marrow cells were placed into culture at low density, a few of them adhered, proliferated, and gave rise to colonies of fibroblast-*like* cells (CFU-f). These adherent fibroblast-*like* cells, but not the hematopoietic cells, when implanted *in vivo*, differentiated into bone tissue and bone marrow stroma, confirming that the bone marrow microenvironment is the niche for two distinct progenitors populations (Friedenstein et al., 1966, 1974a; Owen and Friedenstein, 1988). Cells were then named stromal stem cells. Later, further clonal manipulation and *in vivo* observations led different authors to propose different names, such as mesenchymal stem cells (Caplan, 1991, 2007) and skeletal stem cells (Bianco, 2011), to define almost the same cell population. However, it is important to stress that, even though these names have been used unrestrictedly as synonyms by several different authors, conceptually and originally, they indicate significant differences among the cells, mainly concerning their differentiation potential.

Although several research groups have described different strategies to isolate mesenchymal cells, the CFU-f assay has undergone almost no change since its original description by Friedenstein and coworkers (Friedenstein et al., 1970, 1974b). Higher proliferative rates are usually related to the stem cell and progenitor populations in most normal tissues. It is therefore assumed that each colony of fibroblast-*like* cells (CFU-f) originates from a single stem and/or progenitor cell (Friedenstein et al., 1966, 1974a,b; Latsinik and Epikhina, 1974; Friedenstein, 1976), and the number of colonies observed represents the number of mesenchymal progenitors as a fraction of the number of nucleated cells plated.

For researchers working with mesenchymal cells isolation and expansion, this is the most widely accepted assay used to quantify progenitors numbers. In the present perspective we addressed major topics we believe are most relevant regarding a few specific and distinct aspects of *in vitro* cell adhesion and growth. Even though CFU-f assay is very simple to perform, we proposed three different strategies based on progenitors *in vitro* clonogenic potential, which might be helpful to define standard conditions to optimize *in vitro* manipulation, and provide data linearity and reproducibility.

NUMBER OF CELLS IN A COLONY

In the late 1960's and early 1970's, Alexander Friedenstein and colleagues (Friedenstein et al., 1966, 1970) began their journey into the bone marrow cavity and defined the primary conditions to quantify a sub-population of, by that time, osteogenic progenitors among all bone marrow stromal cells (Friedenstein et al., 1966, 1982). In the original protocol, single cell suspensions of bone marrow cells are plated at low-density (10^4–10^5 nucleated cells per cm^2) and incubated in DMEM supplemented with fetal bovine serum (FBS). Seventy-two hours later, the non-adherent cells are washed out and the adherent fraction is incubated in fresh culture medium. Culture medium is renewed every 3–4 days over a ten-day culture. After a total of 13 days, the cells are fixed and further stained in crystal violet, and the colonies are counted (Satomura et al., 2000; Kuznetsov et al., 2009). It is assumed in this case that when bone marrow cells are plated in low-density cultures, the colonies will not reach each other's borders supporting that each colony is derived from a single progenitor. However, it is important to keep in mind that colonies are heterogeneous and, although each one is derived from a single progenitor, not all display a multilineage differentiation potential. Many are already committed to a specific lineage, following the hierarchical-*like* and controlled differentiation cascade (Muraglia et al., 2000; Sarugaser et al., 2009; Russell et al., 2010). Colonies also display different sizes and cell distribution within the cultures, which may correlate to cell differentiation stage (**Figure 1**). Additional studies have revealed that ∼30% of all BM mesenchymal progenitors colonies present trilineage potential—osteogenic, chondrogenic, and adipogenic—*in vitro*. Sacchetti and coworkers (Sacchetti et al., 2007) demonstrated that ∼50% of the $CD146^+$ clonal mesenchymal progenitors isolated from the bone marrow cultures give rise to compact bone, but not bone marrow, when implanted *in vivo*, indicating that half of the progenitors, upon isolation from an adult bone marrow, are already committed to the osteogenic lineage.

As for several tissues, cells are classified into three categories: (1) stem cells, (2) intermediate progenitors and (3) differentiated cells. Typically, differentiated cells possess low proliferation ability *in vitro*, while intermediate progenitors present high proliferative rates under stimulus. On the other hand, stem cells are quiescent cells *in vivo*, but *in vitro*, under the proper culture conditions, exit the quiescence stage and become highly proliferative (Stanley et al., 1971; Urabe et al., 1979; Nicola and Metcalf, 1986; Oh and Humphries, 2012; Bianco et al., 2013). When analyzed in this perspective, it is expected that several stromal cell populations adhere to the culture flask surface in the first three days of culture, namely differentiated reticular cells, committed progenitors and stem cells. Only progenitors and stem cells will proliferate to generate colonies. Regardless progenitors commitment, what features each group of cells must present to be identified as a colony? Based on the original protocol and refinements suggested by several laboratories (Wagner et al., 2009; Bianco et al., 2013), only colonies consisting of more than 50 cells should be classified as a colony (Kuznetsov et al., 2009). Colonies quantification can be performed under the microscope, but stained colonies with more than 50 cells are easily observed directly by the "naked eye." It must be acknowledged that growth-promoting activity will vary from FBS lot to lot, which may change CFU-f results. Thus, a quality control should be used to screen FBS lots to avoid suboptimal or "superoptimal" conditions, which may result in changes in colony formation and impact cell growth and differentiation (Mannello and Tonti, 2007).

It is clear that cell proliferation status changes accordingly as FBS is changed, which will considerably impact CFU-f results. Cell proliferation depends upon growth factors concentration and, regardless all the controls applied to serum fabrication, this varies largely from FBS lot to lot. Conversely, it is not clear how progenitors respond to serum variations. It must be determined if proliferation of progenitors *in vitro* follows a simultaneous or selective growth pattern, the latter being all

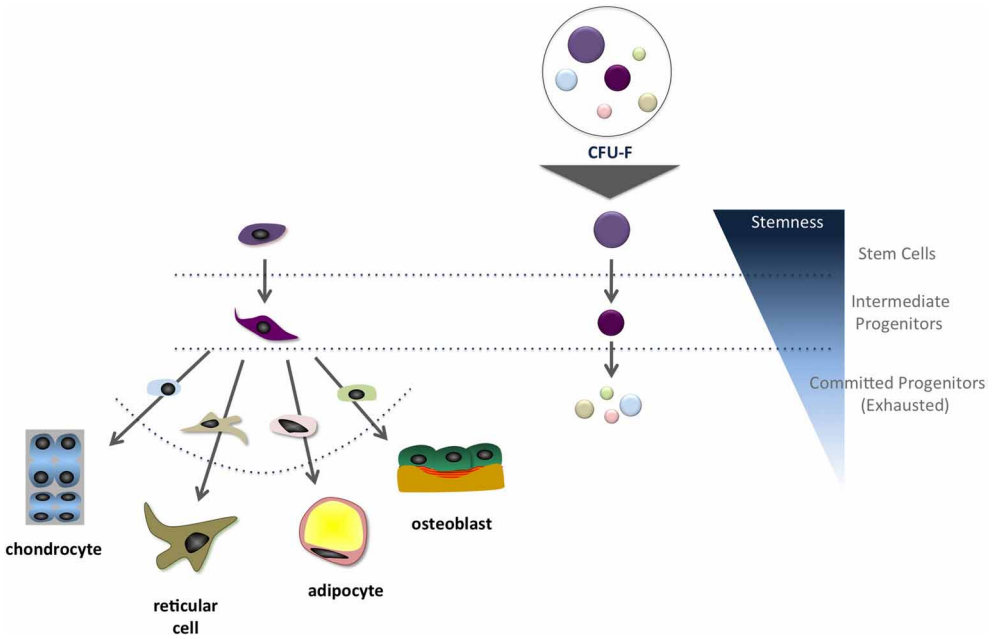

FIGURE 1 | Schematic representation of the suggested correlation between the mesenchymal stem cell differentiation cascade and colony size *in vitro*. It is frequently inferred that most primitive progenitors give rise to larger colonies compared to those originated from intermediate and committed progenitors.

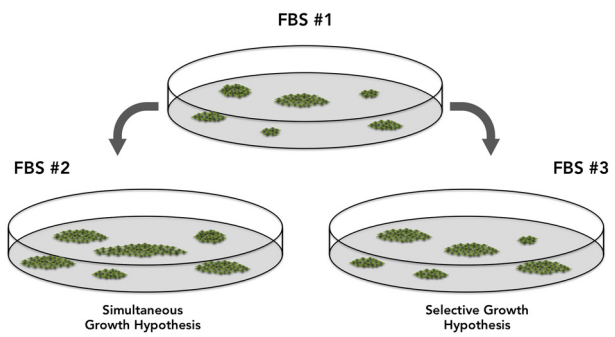

FIGURE 2 | Two hypotheses discussing the potential differences in the Colony Forming Efficiency results associated with the usage of different fetal bovine serum lots. The "Simultaneous Growth Hypothesis" suggests colonies are similarly affected and respond simultaneously, proliferating more or less, but in the same proportion. The "Selective Growth Hypothesis," suggests that in response to different FBS lots, a few progenitors will proliferate more than others, as a result of growth factors concentration and combination.

progenitors will respond differently when FBS lot is changed (**Figure 2**). It is expected that all progenitors in a plate respond similarly (Simultaneous Growth Hypothesis) and, as the FBS lot is changed, they all may proliferate more or less. It is unclear if this is what occurs *in vitro*. Different from the concept that all colonies will be bigger or smaller, the Selective Growth Hypothesis proposes that big colonies might get bigger and small clusters might even not reach the colony status.

At this point, one should know that colony size (the number of cells in a colony) will matter, but only under optimized conditions. To avoid discrepancies and ensure reproducibility, the ideal condition would be to run the CFE assay under chemically defined and controlled culture medium conditions, using recombinant growth factors instead of serum, but this can be cost prohibitive. Moreover, what combination of factors is necessary to expand mesenchymal progenitors *in vitro* remains to be defined. We propose, however, that colony number, colony size, and progenitors phenotype be tested every time serum change is necessary.

DOES COLONY SIZE RELATE TO STEMNESS?

Colony forming ability is not exclusive to the mesenchymal system (Queensberry et al., 1974; Dexter, 1979; Nicola and Metcalf, 1986). A similar *in vitro* assay has been widely used to quantify hematopoietic progenitor cells. In the past, this was the only quantification tool, but it was replaced by the development of more meticulous flow cytometric phenotyping methods, which is now mostly utilized to quantify and identify hematopoietic stem cells and progenitors, although the colony assay remains widely used. It must be clear, however, that cell transplantation into myeloablated animals is the only way to fully identify *bona fide* long-term hematopoietic stem cells (Morrison et al., 1995; Gazit et al., 2008).

Hematopoietic stem cells and progenitor colony forming ability is observed when cells are cultured in semi-solid culture medium, which maintains them in close proximity to each other, as they proliferate in the presence of specific growth factors (Queensberry et al., 1974; Dexter, 1979; Nicola and Metcalf, 1986). Unlike the CFU-f assay, which is used to quantify MSCs and committed progenitors indiscriminately, the colony forming assay can be used to quantify lymphoid, erythroid, myeloid,

and multipotent progenitors separately depending on the combination of specific growth factors added to the culture system (Stanley et al., 1971; Dexter, 1979; Urabe et al., 1979; Nicola and Metcalf, 1986; Quesenberry et al., 1987). Hematopoietic progenitors grow at different culture rates and present distinct morphologies *in vitro*, allowing identification and quantification of different progenitors separately and quickly.

One major observation from the hematopoietic colony-forming assay is that committed progenitors start to proliferate early (day 1 of culture), and develop into distinct colonies in 5–10 days. On the other hand, most primitive progenitors can take a few days to exit the quiescent stage and will eventually form full colonies after 9–14 days in culture (Stanley et al., 1971; Dexter, 1979; Urabe et al., 1979; Nicola and Metcalf, 1986; Quesenberry et al., 1987). Although primitive progenitors take longer to form colonies, the colonies generated are typically 2–4 times larger than those derived from committed progenitors. This *in vitro* behavior is in full accordance with *in vivo* observations, as hematopoietic stem cells are typically quiescent however, when properly stimulated, achieve higher proliferative rates compared to other progenitors (Carow et al., 1993; Ponchio et al., 1995; Hao et al., 1996; Petzer et al., 1996; Oh and Humphries, 2012). For these reasons it is often inferred that the larger the colony, the more primitive the progenitor it had been derived from.

A similar line of observation could be applied to the colony-forming unit fibroblast assay. In a 13-day culture, the committed progenitors will initiate proliferation faster *in vitro* giving rise to colonies, but these cells will rapidly undergo clonal exhaustion. Conversely, uncommitted progenitors will take longer to activate the proliferation cascade, and do not arrest during the culture period and thus result in larger colonies. Indeed, it is not unusual to correlate colony size with primitiveness of the cell of origin.

A question remains unanswered: "Is thirteen days sufficient for all multipotential stem cells to enter the cell cycle and become highly proliferative cells *ex vivo*?" Primitive stem cells are likely to remain deeply quiescent or proliferate very slowly *in vivo*. When placed *in vitro*, they may require longer to leave the quiescent stage but, once stimulated, their proliferative rate is likely to increase beyond that observed for intermediate progenitors. Many hypothesize that the most quiescent mesenchymal stem cells might take longer to start proliferation *in vitro*, which means that a few small/medium colonies may not be representative of exhausted committed progenitors, but rather indicate yet-to-proliferate stem cells (**Figure 3**). This possibility challenges many assumptions derived from the CFU-f assay, and suggests that colony size may not necessarily reflect primitiveness. Cells in colonies derived from committed progenitors are usually large stellate-*like* cells, while in colonies derived from stem cells are often small and fusiform (Gothard et al., 2013). In this case, cell morphology may be a useful tool to tell apart exhausted or expanding small- and medium-sized colonies.

As discussed above, growth factor concentrations in FBS differs among serum lots, and does have significant outcomes on CFU-f size, frequency and even colony morphology. Viewed in terms of the Selective Growth Hypothesis (**Figure 2**), distinct categories of mesenchymal progenitors may be dependent upon different growth factors (and concentrations) to proliferate (or remain quiescent) *in vitro*.

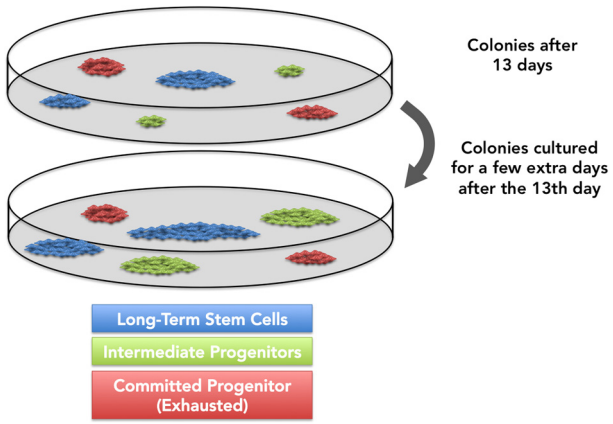

FIGURE 3 | It is generally accepted that most primitive progenitors, upon exiting the lag growth phase, will reach higher proliferation rates than committed progenitors. However, recent data indicate that a few progenitors might require a longer time to enter replicative cycles *in vitro*. We and others hypothesize that, if cultured longer, these progenitors might originate larger colonies.

PROGENITORS ADHESION *IN VITRO*

Previously published data imply that all human bone marrow-derived mesenchymal stem cells and progenitors with the ability to form fibroblast-*like* cell colonies adhere within 2–48 h *in vitro*, followed by a fetal bovine serum-dependent growth (Friedenstein, 1976; Castro-Malaspina et al., 1980; Kastrinaki et al., 2008; Kuznetsov et al., 2009). After this period of incubation, non-adherent cells are discarded and adherent cells are further expanded for at least 10 additional days. The original protocol designed by Friedenstein and coworkers, and corroborated by several other laboratories (Friedenstein et al., 1966, 1970; Owen and Friedenstein, 1988), established the requirement for a 72-h adhesion period prior to the elimination of non-adherent cells. This adhesion period is still considered a critical step for isolation and expansion of these progenitors. It has been described that bone marrow stromal cells must adhere to a substrate *in vitro* for their survival and proliferation, regardless their differentiation potential (Bruder et al., 1997; Pittenger et al., 1999; Dominici et al., 2006). Notwithstanding, authors demonstrate that stromal progenitor cells, or at least a subset of them, can be maintained in stirred suspension cultures for 21 days, and might even proliferate when induced by a combination of cytokines and growth factors (Baksh et al., 2003). Which specific stromal progenitors subsets remain in suspension are yet to be determined.

The average colony forming efficiency for normal human adult bone marrow may vary from 1 to 30 per 1×10^5 nucleated marrow cells (Beresford et al., 1994; Oreffo et al., 1998; Doucet et al., 2005; Bernardo et al., 2007; Kuznetsov et al., 2009). As previously discussed (Kuznetsov et al., 2009), such different values might be the result of either distinct cell isolation/preparation procedures or cell culture conditions, the latter meaning FBS capacity to induce progenitors proliferation *in vitro*. Although proliferation status has been a very useful tool to evaluate FBS quality, progenitors adhesion capacity, not usually considered, is crucial for colony forming efficiency and does affect the results.

It is not clear, however, if and how different concentrations of growth factors and cytokines in FBS would impact progenitors adhesion *in vitro*. Most recently, Di Maggio and coworkers (Di Maggio et al., 2012) demonstrated that highly proliferative multipotent progenitors can be isolated from the non-adherent fraction, after 3 days, when bone marrow cells are cultured in the presence of FGF2. In addition to previous observations (Baksh et al., 2003), these data indicate that mesenchymal progenitors, or at least a subset, might change its adhesion properties accordingly as growth factors and cytokines concentration change in FBS. Currently, it is difficult to precise how much time mesenchymal stem cells and progenitors need to adhere *in vitro*, and how it is influenced by FBS growth factors concentrations. Further studies will be necessary as progenitors adhesion represents one crucial step when it comes to mesenchymal progenitors isolation.

PERSPECTIVE

The fibroblast-*like* colony-forming assay is simple to perform: cells are plated; progenitors adhere to the substrate and proliferate in a short culture period; cells are fixed and stained; colonies with more than 50 cells are counted. The number of progenitors in a given cell population can be easily approximated. Colony-forming efficiency results, however, depend upon an optimized procedure and several steps must be optimized to provide linear and reproducible data. Herein, our purpose was to revisit this classic method and open a discussion based on three issues to assist assay performance and data interpretation, but mostly to help researchers establish in their labs a standardized procedure. It is critical to optimize the ability of the cells to proliferate in response to growth factors present in the FBS, or any other source used, and this is commonly neglected due to the "quantification-only" use of the method. As a renewable source for tissue regeneration, *in vitro* cell expansion is almost always required for MSC populations, meaning that these cells will not be used as primary cultures, but only after, at least, 3 passages. It would be expected that after several rounds of expansion, most primitive progenitors and stem cells would take over the culture at the expense of other progenitors. Nevertheless, this has not been well elucidated so far and it is not known how mesenchymal population responds to different growth factors concentration in different serum lots. Although several research groups have described different strategies MSCs, the cell populations obtained and expanded remain heterogeneous in terms of stem cells and committed progenitors regardless of the method used for isolation (Muraglia et al., 2000; Sacchetti et al., 2007). No current method ensures isolation of a pure population of MSCs, or even whether this is desirable but this fact, the lack of standards, makes difficult understanding of the biology and true potential of these cells.

For these reasons it would be interesting that researchers establish a "surveillance system" when working with mesenchymal progenitors isolation and expansion. We therefore propose a list of three procedures recommended as controls. First, it would be highly desirable for each laboratory to keep a standard collection of bone marrow samples to be used as references for testing serum lots. Conceptually, each lab could select representative bone marrow samples, split each of them into equal aliquots (10, 20, 30, or as many as possible), freeze all at the same time, and cryopreserve (**Figure 4**). These aliquots will be used to test serum "quality" every time changes of lots are necessary.

As a second control instrument, the influence of FBS in progenitors adhesion should be evaluated, regarding colony forming efficiency (**Figure 5**). In one group (we suggest triplicate), bone marrow cells are incubated in culture medium supplemented with control serum. After 72 h, non-adherent cells are discarded and adherent cells are cultured for 10 additional days also in culture medium supplemented with control FBS. In another group, bone marrow cells are incubated in culture medium supplemented with testing serum. After 72 h, non-adherent cells are discarded and adherent cells are culture for 10 additional days in culture medium supplemented with control FBS. The main objective of the assay is to analyze if different serum lots contribute to colony forming efficiency change at the expense of cell adhesion or cell proliferation.

Mesenchymal progenitors' self-renewal capacity can be tested by evaluating the ability of expanded clones to originate secondary colonies upon replating. As a third parameter, we propose the secondary colony forming efficiency assay (**Figure 6**). One thousand cells from the primary colony-forming cultures may be replated and cultured for additional 10 days. Cells are, then, fixed and stained. It is important to highlight that, in all three assays colony numbers, and colony size must be rigorously evaluated and compared.

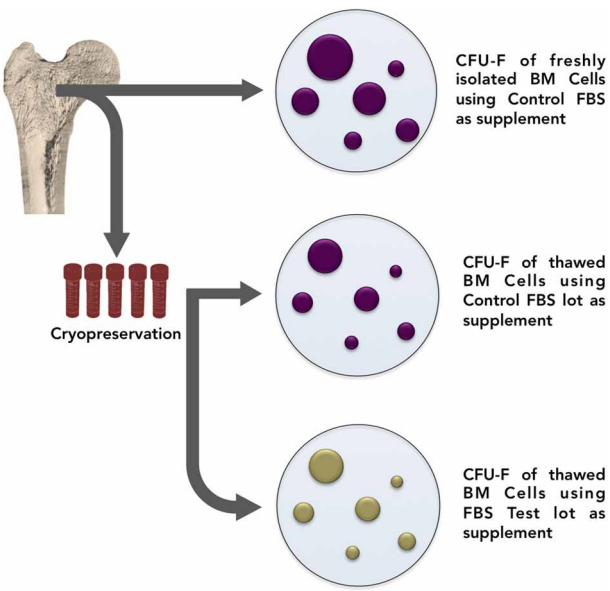

FIGURE 4 | In order to establish a "surveillance system" when working with mesenchymal progenitors isolation and expansion, it is suggested that each laboratory keep a standard collection of bone marrow samples to be used as references for testing serum lots. Conceptually, each lab could select representative bone marrow samples, split each of them into equal aliquots (10, 20, 30, or as many as possible), freeze all at the same time, and cryopreserve. These aliquots will then be thawed and used to test serum "quality" every time changes of lots are necessary. From time to time, bone marrow collection must be renewed.

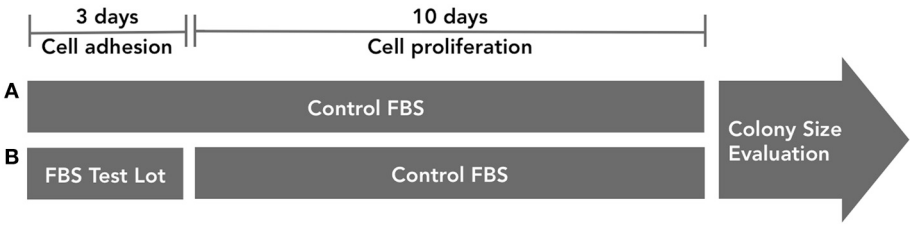

FIGURE 5 | FBS influence on progenitor cell adhesion *in vitro* must be tested. In group **(A)**, bone marrow cells are incubated in culture medium supplemented with control serum. After 72 h, non-adherent cells are discarded and adherent cells are cultured for 10 additional days also in culture medium supplemented with control FBS. In group **(B)**, bone marrow cells are incubated in culture medium supplemented with testing serum. After 72 h, non-adherent cells are discarded and adherent cells are culture for 10 additional days in culture medium supplemented with control FBS. Colony numbers and size must be evaluated.

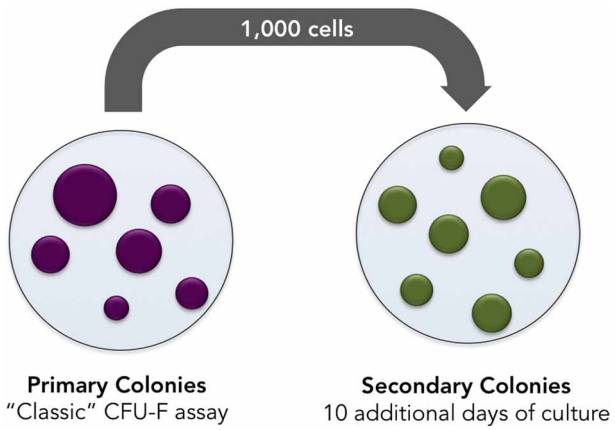

FIGURE 6 | Secondary colonies assay. Bone marrow cells are incubated in culture medium supplemented with control or testing FBS. Seventy-two hours later, non-adherent cells are discarded and adherent cells are cultured in culture medium supplemented with control or testing serum for 10 additional days. Later, cells are tripsinized and single-cell suspension prepared. One thousand cells are subsequently replated and incubated for 10 additional days in culture medium supplemented with testing or control serum. Numbers and size of secondary colonies must be evaluated.

It is clear that cell behavior *in vivo* and *in vitro* changes from person to person, from animal to animal, even among syngeneic siblings, and over time in the same individual. We propose that these three strategies combining in-colony proliferation rate analyses with colonies formation and quantification, progenitors adhesion, and recloning ability, if used as a lab surveillance instrument, will assist in the development of a greater understanding of the biology of MSCs and other adherent populations *in vitro*. We would like to propose the creation of control guidelines which will facilitate the identification and isolation of long-term stem cells and short-term progenitors as a crucial step to better explore their potential and define their applicability in the cell therapy and bioengineering fields.

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Growth Factor Content in Human Sera Affects the Isolation of Mesangiogenic Progenitor Cells (MPCs) from Human Bone Marrow

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Mesangiogenic Progenitor Cells (MPCs) are human bone marrow-derived multipotent cells, isolated *in vitro* under selective culture conditions and shown to retain both mesengenic and angiogenic potential. MPCs also co-isolated with multipotent stromal cells (MSCs) when bone marrow primary cultures were set up for clinical applications, using human serum (HS) in place of fetal bovine serum (FBS). MPC culture purity (over 95%) is strictly dependent on HS supplementation with significant batch-to-batch variability. In the present paper we screened different sources of commercially available pooled human AB type serum (PhABS) for their ability to promote MPC production under selective culture conditions. As the majority of "contaminating" cells in MPC cultures were represented by MSC-like cells, we hypothesized a role by differentiating agents present in the sera. Therefore, we tested a number of growth factors (hGF) and found that higher concentrations of FGF-2, EGF, PDGF-AB, and VEGF-A as well as lower concentration of IGF-1 give sub-optimal MPC recovery. Gene expression analysis of hGF receptors was also carried out both in MSCs and MPCs, suggesting that FGF-2, EGF, and PDGF-AB could act promoting MSC proliferation, while VEGF-A contribute to MSC-like cell contamination, triggering MPC differentiation. Here we demonstrated that managing hGF contents, together with applying specific receptors inhibitors (*Erlotinib*-HCl and *Nintedanib*), could significantly mitigate the batch-to-batch variability related to serum supplementation. These data represent a fundamental milestone in view of manufacturing MPC-based medicinal products.

Keywords: mesangiogenic progenitor cells, mesenchymal stromal cells, human serum, growth factors, bone marrow culture, cell-based medicinal product, *Erlotinib*, *Nintedanib*

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INTRODUCTION

Mesenchymal stromal cells (MSCs) are multipotent adult progenitor cells isolated *in vitro* from a plethora of different tissues (reviewed in Murray et al., 2014) as their perivascular origin has been demonstrated and widely accepted (da Silva Meirelles et al., 2006; Crisan et al., 2008). MSC role as skeletal tissue progenitors has been investigated in a variety of studies in animal models, and cell-based therapy approaches demonstrated their beneficial effects in the regeneration of damaged

skeletal tissues including bone (Watson et al., 2014), cartilage (Richardson et al., 2015), tendons (Pacini et al., 2007), and meniscus (Yu et al., 2015). For those reasons, most of the tissue engineering strategies and regenerative medicine approaches, developed to *de novo* skeletal tissue formation, involved MSCs (Yousefi et al., 2016). Nonetheless, it has been reported that an effective tissue remodeling response is in tight correlation with the formation of vascularized tissue (Rao and Stegemann, 2013; Hutton and Grayson, 2014). Commonly, the vascularization of the newly formed tissues *in vivo* has been expected driven in large part by the host response to the implant, and trigged by hypoxic condition surrounding the grafted cells (Chamberlain et al., 2015). Nonetheless, this effect could be hypothesized in acute injuries with moderate tissue loss, but in case of large implants or poor blood supply, as in non-union fracture or pseudoarthrosis (Hak et al., 2014), the vascularization of constructs would be compromised. Modular constructs obtained applying both osteoprogenitor cells, as MSCs, and endothelial cells (ECs) or their precursors (EPCs), have been investigated as an option in order to sustain vascularization of the implants, independently from the host response (Butler and Sefton, 2012). However, MSCs have been estimated to represent around 0.001–0.01% of human bone marrow mononuclear cells (hBM-MNCs) (Subbanna, 2007; Lechanteur et al., 2016), and similarly ECs and EPCs represent rare populations in their tissues of origin (Pelosi et al., 2014), thus pre-clinical and clinical applications of these cells, alone or in combination, require extensive *ex vivo* cell expansion to obtain therapeutic cell doses. Most widely accepted MSC isolation and expansion protocols, from bone marrow, are based on basal media supplemented with fetal bovine serum (FBS) (Haynesworth et al., 1992; Prockop, 1997). Similarly, consistent fractions of FBS should be applied also in the EC and EPC isolation (Kirton and Xu, 2010). However, all supplements of animal origin expose patients to a number of risks and represent a major obstacle to comply with good manufacturing practice (GMP) guidelines (Herberts et al., 2011). The urgent need for FBS alternatives led to consider human serum (HS) as the straightforward substitute in clinical-grade MSC production (Altaie et al., 2016).

In 2009, during the attempt to produce multipotent mesenchymal stromal cells (MSCs) in xeno-free GMP-compliant culture conditions, we had shown the occurrence of mesodermal (currently renamed "mesangiogenic") progenitor cells (MPCs), in hBM-MNC cultures when using autologous serum (AS) (Petrini et al., 2009) or pooled human AB type serum (PhABS) (Trombi et al., 2009). MPCs have been described *in vitro* as slow cycling MSC progenitors that also retain angiogenic properties (Fazzi et al., 2011). They are characterized by peculiar fried egg-shape morphology, expression of pluripotency-associated markers *OCT4* and *NANOG*, and intense nestin expression (Pacini et al., 2010). MPCs lack mesenchymal-associated markers CD73, CD90, and CD105 while showing occurrence of CD31, CD11c, and CD18.

After more than 6 years spent on MPC characterization and optimization of isolating procedures, we believe that these cells could facing a new era and possibly being involved in skeletal tissue engineering and regeneration. As MPCs demonstrated *in vitro* retaining both mesengenic and angiogenic potential, it reasonable hypothesizes the development of MPC-based implants in alternative to the modular constructs. The need of two or more different expanded cell populations giving a mesangiogenic potential to the engineered tissues could be overtaken applying cell populations like MPCs (Pacini and Petrini, 2014). However, the definition of a highly reproducible clinical-grade manufacturing process represents a first step in dealing with a possible application of MPCs in cell-based therapies. From the first report of mesangiogenic cells in human bone marrow cultures applying AS, a consistent variability in the MPC harvesting was reported. Only two out of every three samples cultured showed detectable MPC population co-isolated with MSCs, with percentages that could vary 10-fold (Petrini et al., 2009). As a consequence, we believe that the supplementation with human sera could induce considerable culture variability, during cell manufacturing process, depending on donor sex and age as well as diseases and pharmacological treatments at the time of serum collection, especially in the autologous context. Consistent production of almost pure MPC cultures has been achieved applying commercially available PhABS. Under these selective culture conditions it was possible to obtain MPCs from all the samples, with recoveries showing very low coefficient of variation (≈10%). Conversely, culturing the same samples in FBS supplemented media led to cell products constituted by MSCs only (Trombi et al., 2009). Nonetheless, we recently reported that isolation of MPCs at high grade of purity (over 95%) would be possible only after accurate screening of commercially available PhABS (Montali et al., 2016). In fact, a small number of tested batches produced cell products with consistent percentages of MSC-like cell population, compromising the purity of the MPC preparations. In order to define specific sera properties, allowing the optimal MPC manufacturing process, would be helpful the comparisons between PhABS with good performances and PhABS promoting MSC "contamination."

Human serum contains a large and untreatable number of biological active molecules possible affecting the MPC isolation and inducing MSC proliferation (Rodrigues et al., 2010). However, recent efforts in the establishment of serum-free media for MSC *in vitro* expansion, demonstrated that human platelet lysate (hPL) could efficiently replace serum in culture (reviewed in Burnouf et al., 2016). This important finding narrows the choice of essential components to platelet granules content (Fekete et al., 2014). Platelets contain a lot of potent biological active molecules (Semple et al., 2011), mainly stored in the α-granules (Golebiewska and Poole, 2015), in addition to important factors involved in coagulation. These molecules include different chemokines (Semple et al., 2011) and human growth factors (hGFs), such as epidermal growth factor (EGF), basic fibroblast growth factor (FGF-2), platelet derived growth factor isoforms (PDGF-AA, -AB, and-BB), insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF), and some others (Nurden et al., 2008).

In order to define reproducible MPC manufacturing process, in the present paper we moved a first step focusing on the effect of the platelet-derived hGFs, mentioned above, on the MPC

production. EGF, FGF-2, PDGFs, IGF-1, and VEGF have been reported regulating proliferation and survival of BM-derived MSCs. Thus, we hypothesize that these five hGFs or some of them could promote expansion of MSCs, during primary bone marrow cultures intended for MPC production. Here we compare six different commercial PhABS, selected on the basis of their different performance, attempting to correlate the percentage of "contaminant" MSC-like cells with the serum concentration of those hGFs.

MATERIALS AND METHODS

Screening of Human Sera for MPC Isolation

The study has been performed according to the declaration of Helsinki and the sample collection protocol was approved by the ethical committee of "*Azienda Ospedaliero-Universitaria Pisana*". Bone marrow aspirates were obtained after written consent from 8 patients (4M/4F, median age 65), undergoing orthopedic surgery for hip replacement. Soon after femoral neck osteotomy, approximately 10 ml of bone marrow were aspirated, using a 20 ml syringe containing 500 U.I. of heparin, and promptly sent to the cell culture facility. Samples were diluted 1:4 and hBM-MNCs collected by density gradient centrifugation using Ficoll-Paque^{TM}PREMIUM (GE Healthcare, Uppsala, Sweden). After two washes in Dulbecco's Modified Phosphate Buffer (D-PBS, LifeTechnologies, Carlsbad, USA-CA) cells were plated at $8 \times 10^5/cm^2$ in hydrophobic six-well plates for suspension culture (Greiner Bio-One, Kremsmünster, Austria) and cultured in minimal essential medium supplemented with PhABS as previously described (Montali et al., 2016). Briefly, low-glucose Dulbecco's modified Eagle medium (DMEM, LifeTechnologies) was supplemented with 2 mM Glutamax® (LifeTechnologies), 100 μg/mL gentamicin (LifeTechnologies), and 10% PhABS. We tested six different commercially available PhABS (AB type and off-the-clot) from four different manufacturers (**Table 1**). Culture medium was changed every 48 h. After 7–8 days, plates were morphologically screened for MPCs using an inverted microscope, and cells subsequently detached by TrypLE® Select (LifeTechnologies) digestion and washed in D-PBS.

MSC Cell Culture

Duplicate hBM-MNC samples collected by density gradient centrifugation were plated at $2 \times 10^5/cm^2$ in gas-treated culture plates for adherent cells. DMEM was supplemented with 2 mM Glutamax® (LifeTechnologies), 100 μg/mL gentamicin (LifeTechnologies), and 10% FBS (LifeTechnologies). Culture medium was changed after 48 h to remove non-adherent cells and refreshed twice a week.

Flow Cytometry

To identify MPCs and MSC-like cells (Keating, 2012) in MPC primary cultures freshly detached cells were incubated with anti-CD90 FITC-conjugated, anti-CD73 PE-conjugated, anti-CD31 PE/Cy7-conjugated, anti-CD18 APC-conjugated, and anti-CD45 VioBlue® -conjugated (Miltenyi Biotec, BergischGladbach, Germany) antibodies for 30′ at 4°C in the dark and washed twice in MACSQuant® Running Buffer (Miltenyi Biotec). Data were acquired using MACSQuant® flow cytometer and analyzed by MACSQuantify® Analysis Software (MiltenyiBiotec). MPCs were identified as $CD31^+CD18^+CD45^{low}CD73^{neg}CD90^{neg}$ events and MSCs as $CD31^{neg}CD18^{neg}CD45^{neg}CD73^{bright}CD90^{bright}$ events. Statistical analysis was performed by one-way analysis of variance (ANOVA) test and Dunnett's post-test for multiple comparison. Results were expressed as mean value ± standard error (SE).

Gene Expression

MPCs and MSCs from primary cultures were washed twice in D-PBS, and pellets cryo-preserved in liquid nitrogen to be processed. Total RNA extraction was performed soon after thawing, using RNeasyMicro Kit (Qiage, Hilden, Germany) according to manufacturer. RNA samples (100 ng) were retro-transcribed using QuantiTect® Reverse Transcription Kit (Qiagen) and 2 μl samples of 10-fold cDNA dilutions were amplified by quantitative Real Time PCR (qRT-PCR), using iCycler-iQ5 Optical System (Bio-Rad, Hercules, USA-CA) and SsoAdvancedSYBR Green SuperMix (Bio-Rad). Samples were run in duplicate. Primer pairs (Sigma-Aldrich, St. Luis, USA-MO) were designed to detect growth factor receptor genes: *BMPR1A*, *BMPR2*, *EGFR*, *FGFR1*, *FGFR2*, *FGFR3*, *IGF1R*, *IGF2R*, *KDR*, *PDGFRA*, *PDGFRB*, *TGFBR1*, *TGFBR2*, and *TGFBR3* (Supplementary Table S1). Relative quantitative analysis was performed following $2^{-\Delta\Delta Ct}$ Livak method (Livak and Schmittgen, 2001). Normalization was performed by using *RPL13A* and *ACTB* housekeeping genes. Values were reported as log-ratios of mean MPC/MSC normalized fold expression.

TABLE 1 | PhABS details.

Code	Lot#	Manufacturer	Type	Production method	Gender	Origin
LZM	N.R.*	Lonza, Basel, Switzerland	AB	Off-the-clot	Male only	USA
LZMF	N.R.*	Lonza, Basel, Switzerland	AB	Off-the-clot	Not declared	USA
SERL	E8051213	SeraLab, West Sussex, UK	AB	Off-the-clot	Male only	USA
SIGM	SLBF-3954V	Sigma Aldrich, St. Luis, USA	AB	Off-the-clot	Not declared	USA
BIOW1	S10443S4190	BioWest, Nuaillé, France	AB	Off-the-clot	Male only	EU
BIOW2	S10169S4190	BioWest, Nuaillé, France	AB	Off-the-clot	Male only	EU

**N.R., Not Recorded.*

Enzyme-Linked Immunosorbent Assay (ELISA)

Growth factor quantification was performed on 200 μl aliquots of serum batches, by colorimetric solid phase ELISA. In particular; epidermal growth factor (EGF), fibroblast growth factor-2 (FGF-2), and platelet-derived growth factor AB (PDGF-AB) were quantified using Quantikine® immunoassay kit (R&D Systems, Minneapolis, USA-MN). Vascular endothelial growth factor A (VEGF-A) was quantified by VEGF-A (human) BioLISA Kit Bender MedSystems® (Vienna, Austria) and insulin-like growth factor-1 (IGF-1) by IGF-1 600 ELISA kit from DRG International (Marburg, Germany). Assays were performed according to manufacturer. Percentages of MSC-like cells in MPC primary cultures were correlated with growth factor concentrations in specific sera batches by Spearman's correlation.

Recombinant hGF Treatments during MPC Isolation

hBM-MNCs were obtained from 4 patients (2M/2F, median age 68) and processed for MPC isolation as described above, applying two different sera batches: LZM, already screened for good performance and BIOW2 that produced cultures with consistent MSC-like cell "contamination." LZM cultures were also performed in presence of 50 ng/ml rhEGF (ThermoFisher Scientific, Waltham, MA USA), 20 ng/ml rhFGF-2 (ThermoFisher Scientific), 50 ng/ml rhPDGF-AB (PeproTech EC Ltd., London, UK) or 50 ng/ml rhVEGF (ThermoFisher). Combined treatments were also performed adding rhEGF and rhFGF-2, or rhVEGF and rhPDGF-AB. In parallel BIOW2 cultures were also supplemented with 100 ng/ml rhIGF-1 (PeproTech EC Ltd., London, UK), 30 ng/ml rhIGF-2 (PeproTech EC Ltd.) alone or in combination. The culture media were changed every 2 days restoring concentrations of the hGFs. After 7 days cells were detached by TrypLE® Select digestion and processed for flow cytometry to quantify MPC and MSC percentages in the cultures, as described above. Data were collected in duplicate and reported as median values ± standard error (SE). One-way ANOVA test, coupled with Dunnett's post-test, was applied to identify significant differences.

Inhibition of hGF Receptors, during Primary Cultures

Further six bone marrow samples (4M/2F median age 68) were processed, as described above, to obtain MPC culture in BIOW2 sera batch with or without adding 4 nM of *Erlotinib*-HCl (OSI-744, SelleckChem, Houston, USA-TX), a potent EGFR inhibitor, and 100 nM of *Nintedanib* (BIBF 1120, SelleckChem) to simultaneously inhibit VEGF, FGF, and PDGF receptors. Cell cultures were performed in duplicate in six-well culture plates for suspension cultures, media were changed after 48 and 72 h restoring inhibitors concentration and at day 6 cultures were detached for flow cytometry analysis to quantify mesenchymal $CD73^{+}CD90^{+}$ population. Similarly and in parallel, MPC cultures were performed applying LZM sera batch in presence or absence of 50 nM of *Linsitinib* (OSI-906, SelleckChem) as IGF-1R inhibitor. Significant differences were revealed by one-way ANOVA test, and the inhibition index was calculated as difference in $CD73^{+}CD90^{+}$ percentages in treated and no-treated cultures divided by the percentage in the non-treated. Data were collected in duplicate and reported as median values ± standard error (SE).

Inhibition of hGF Receptors, during Mesengenic Differentiation of MPCs

Erlotinib-HCl and *Nintedanib* were also applied during mesengenic differentiation of MPCs. Briefly, MPC culture in LZM sera batch were validated for a purity higher than 95% and re-plated at 20,000 cells/cm^2 in gas-treated 6-well plates for adherent cultures. After overnight incubation medium was replaced with StemMACS™ MSC expansion XF medium (Miltenyi Biotec), according to previously reported protocol (Montali et al., 2016). In parallel, mesengenic differentiation was performed in presence of 4 nM of *Erlotinib*-HCl and 100 nM of *Nintedanib*. Cultures were maintained changing medium twice a week and restoring inhibitor concentration. After 7 days mesengenic differentiation was evaluated by AlamarBlue® reduction assay (LifeTechnologies) as previously reported (Fazzi et al., 2011).

RESULTS

We were able to isolate and grow MPCs from hBM-MNC primary cultures supplemented with all six PhABS under test. Cell yields were adequate ($1.20 \pm 0.23 \times 10^5$ cells/well, $n = 8$) to perform further analysis. Cultures supplemented with *Lonza* PhABS from males (LZM) or from males and females (LZMF) showed to be constituted almost exclusively by cells with the typical fried egg-shape morphology that identifies MPCs (**Figure 1A**; Petrini et al., 2009). Flow cytometry confirmed the characteristic $CD31^{+}CD18^{+}CD45^{low}CD73^{neg}CD90^{neg}$ MPC phenotype for over 95% of the cell population (red dots in **Figure 1A**) beside a very small $CD31^{neg}CD18^{neg}CD45^{neg}CD73^{bright}CD90^{bright}$ population of MSC-like cells (blue dots in **Figure 1A**; Montali et al., 2016). Similar results were obtained using *SeraLab* PhABS (SERL) (**Figure 1A**). *Sigma-Aldrich* PhABS (SIGM) supplementation resulted in the presence of occasional spindle-shaped MSC-like cells together with a mild increase in the percentage of MSC immunophenotype (**Figure 1A**). Cultures supplemented with two lots of *BioWest* PhABS (BIOW1, BIOW2) showed a large population (10–30%) of MSC-like cells, co-isolated with MPCs. Most MSC-like cells were organized in clusters or in colonies (white arrows in **Figure 1A**).

We previously defined the cut point for MPC cell production as 95% of $CD31^{+}CD18^{+}CD45^{low}CD73^{neg}CD90^{neg}$ cells within MPC primary cultures (Montali et al., 2016). Three out of the six serum batches resulted suitable for MPC production, showing percentages of MSC-like cells lower than 5% with no significant variation: LZM (2.7 ± 0.6%), LZMF (3.3 ± 0.5%), SERL (3.3 ± 1.2%). SIGM was borderline (5.9 ± 1.4%), while BIOW1 and BIOW2 turned out not to be fitting because of significantly ($p <$

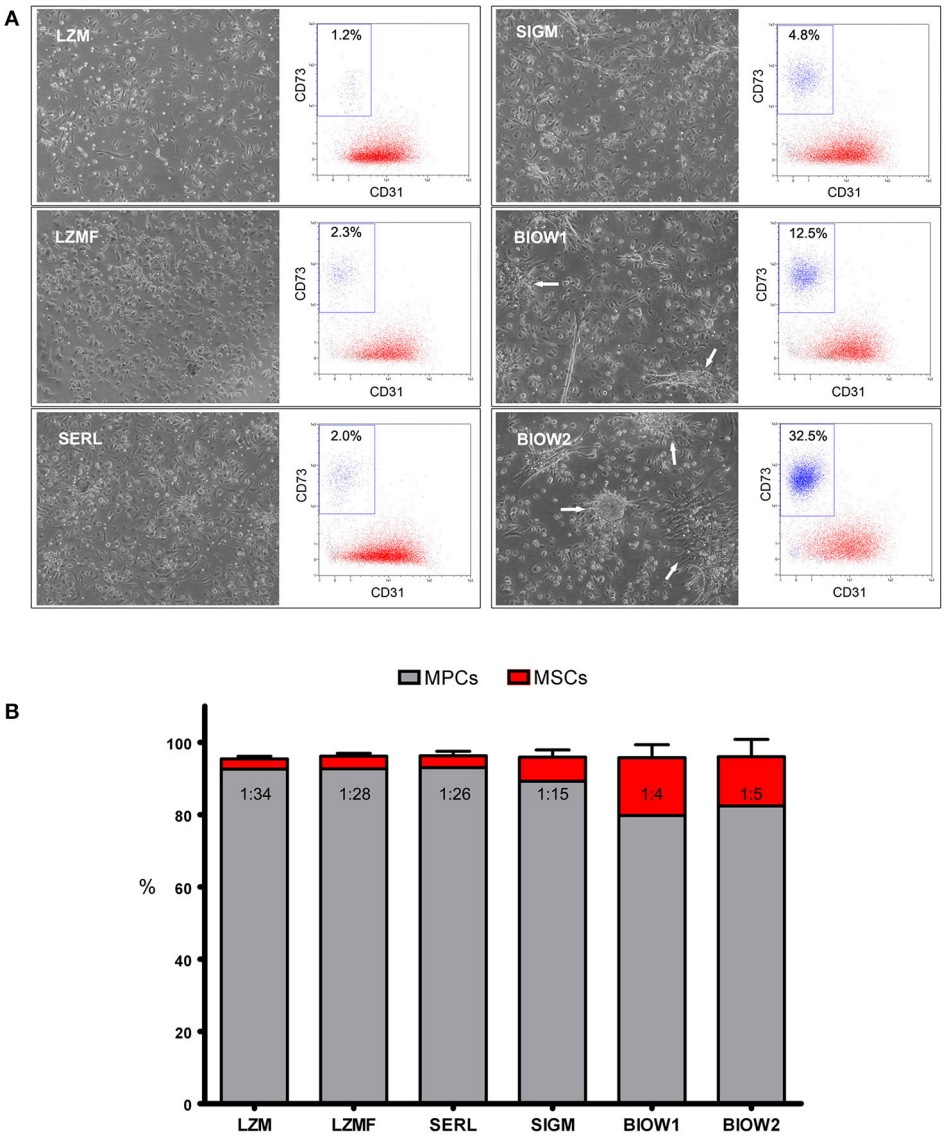

FIGURE 1 | Analysis of different PhABS in MPC cultures from bone marrow. (A) Six different PhABS were tested for their efficiency in the isolation of MPCs. After 6 days of culture, LZM, LZMF, and SERL originated MPC cultures at high grade of purity. Cultures supplemented with SIGM showed rare MSC-like cells, while BIOW1 and BIOW2 produced a number of MSC-like cells usually clustered in proliferating foci or in rounded colonies (white arrows). Purity of cultures was estimated by flow cytometry. $CD73^{bright}CD90^{bright}CD31^{neg}$ (blue dots) percentages lower than 5.0% accounted for a good performance in MPC (red dots) production. **(B)** Mean MSC/MPC ratio in LZM, LZMF, and SERL varied from 1:26 to 1:34, as opposed to 1:4 and 1:5 detected in SIGM, BIOW1, and BIOW2.

0.01 to LZM, $p < 0.05$ to LZMF and SERL) higher percentages of MSC-like cells (17.7 ± 4.8% and 16.2 ± 3.9%, respectively) resulting in MSC/MPC frequencies around 1:4–1:5 (**Figure 1B**).

MPCs from LZM supplemented cultures were selectively chosen for gene expression analysis of growth factor receptors in comparison to standard FBS-cultured MSCs. Expression of 3 out of the 14 genes analyzed was over one log higher in MSCs: *FGFR2* (−1.74 log, $p < 0.05$, $n = 8$), *PDGFRA* (−1.58 log, $p < 0.05$, $n = 8$), and *EGFR* (−1.33 log, $p < 0.01$, $n = 8$). *PDGFRB* and *TGFR3* were also significantly more expressed in MSCs although at lower levels (−0.90 and −0.69 log, respectively, $p < 0.05$, $n = 8$). Conversely, *IGF2R* and *KDR* expression was significantly higher in MPCs, with *IGF2R* only over one log higher (1.27 log, $p < 0.01$, $n = 8$, **Figure 2**). Higher levels of *IGF1R* and *TGFRB2* were detected in MPCs with a confidence limit lower than 90% (0.37 log, $p = 0.373$ and 0.51 log, $p = 0.198$, $n = 8$, respectively). *BMPR1A*, *BMPR2*, *FGFR1*, *FGFR3*, and *TGFRB1* showed no significant differences.

The "contamination" of MSC-like cells in MPC primary cultures was correlated to the concentration of growth factors in the different sera. Higher concentrations of EGF, FGF-2, PDGF-AB and VEGF-A were consistently found in BIOW1

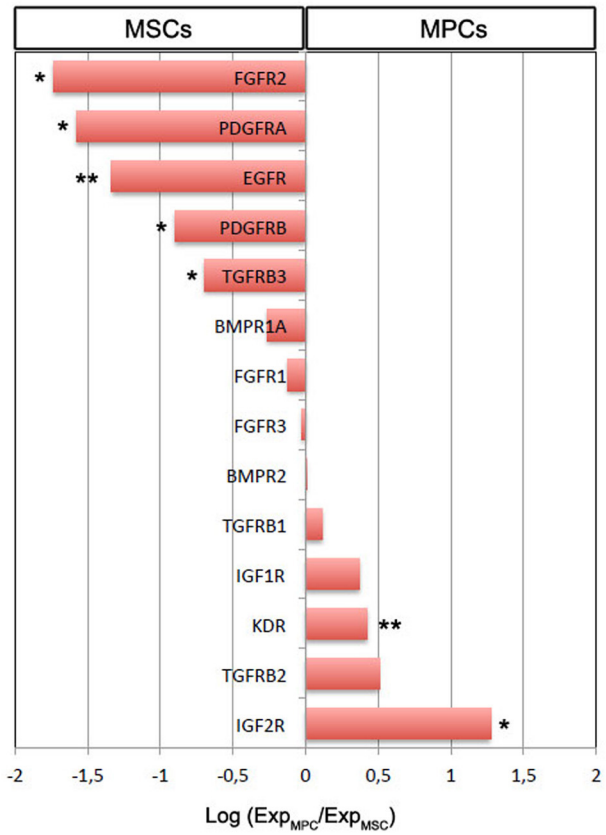

FIGURE 2 | Gene expression analysis of growth factor receptor genes in MSCs vs. MPCs. The majority of the receptor genes resulted differentially expressed (*$p < 0.05$, **$p < 0.01$). Expression of *FGFR2*, *PDGFRA*, and *EGFR* was over one log higher in MSCs. Conversely, 10-fold higher *IGF2R* expression was evidenced in MPCs.

and BIOW2 that produced cultures with higher percentages of MSC-like cells (**Table 2**). FGF-2 revealed a dose-dependent correlation showing a Spearman ratio of 0.943 ($p < 0.05$, $n = 8$, **Figure 3**). In order to confirm the involvement of these hGFs in the poor performance of a pooled sera batch, we separately added consistent amount these factors to LZM supplemented cultures. Percentages of MSC-like cells resulted significantly increased in the rhEGF (34.3 ± 14.0%, $p < 0.01$, $n = 4$) and rhFGF-2 (39.2 ± 17.5%, $p < 0.01$, $n = 4$) treated primary cultures, respect to LZM control (4.6 ± 1.7%, $n = 4$, **Figure 4**). Combining these two hGFs produced similar results (40.5 ± 17.9%, $n = 4$), with no significant differences respect to cultures treated with EGF or FGF-2 separately. Conversely, adding rhVEGF (5.6 ± 1.4%, $n = 4$) or rhPDGF-AB (6.4 ± 0.5%, $n = 4$) resulted having no significant effects on MPC cultures when added separately. Combination of those hGF instead showed a mild increase in MSC-like cell percentages (11.3 ± 0.3%, $p < 0.01$, $n = 4$), resulting in cultures with purity lower than the cut-off point established for feasible MPC products.

Medium supplemented with BIOW2 was selected in order to verify if higher concentration of IGFs could be related to the good performances of feasible sera batches. A consistent reduction,

TABLE 2 | PhABS growth factor concentrations.

PhABS batch	EGF (pg/ml)	FGF-2 (pg/ml)	PDGF-AB (pg/ml)	VEGF-A (pg/ml)	IGF-1 (ng/ml)
LZM	44	0.3	584	80	205
LZMF	154	2.0	1223	175	157
SERL	7	0.5	564	78	141
SIGM	42	1.1	584	78	167
BIOW1	263	7.6	1943	440	114
BIOW2	261	5.7	2000	495	132

around 50%, of the MSC "contamination" was reported in rhIGF-1 (17.1 ± 0.1%, $p < 0.01$, $n = 4$) and rhIGF-2 (17.7 ± 0.2%, $p < 0.01$, $n = 4$) treated cultures, alone or in combination (19.8 ± 2.8%, $p < 0.01$, $n = 4$) respect to BIOW2 control (45.8 ± 1.6%, $n = 4$, **Figure 4**).

The effect of high concentration of EGF, FGF-2, VEGF and PDGFs in the poor performance of pooled sera batches, during MPC production, was also confirmed by receptor inhibition experiments. In fact, the addition of *Erlotinib*-HCl and *Nintedanib* to BIOW2 cultures resulted in the 50% reduction of the $CD73^{+}CD90^{+}$ population (mean inhibition index: 54.2 ± 7.7%, $p < 0.05$, $n = 6$) at the primary cultures, producing suitable MPC products (>95% of purity) comparable to ones obtained applying LZM (**Figure 5A**). Elevated levels of IGF-1 were instead detected in the sera batches with low mesenchymal contamination (LZM, LZMF) but the inhibition of its receptors did not produce any detectable effect during primary culture (**Figure 5B**).

Erlotinib-HCl and *Nintenadib* also affected the *in vitro* MPC mesengenic differentiation, with a decreased AlamarBlue® reduction from 20 to 40% respect to control (mean inhibition index: 22.7 ± 10.9%, $p < 0.05$, $n = 6$). Conversely, *Linsitinib* produced no detectable effects (data not shown).

DISCUSSION

Ex vivo expansion of bone marrow-derived multipotent cells requires supplementation of basal culture medium in order to sustain cell proliferation while maintaining cell differentiation capability (Bernardo et al., 2011). FBS-based expansion protocols have been used in the very first clinical applications of these promising cells (Le Blanc et al., 2004, 2008). However, safety concerns have been raised regarding use of supplements of animal origin in clinical-grade expansion protocols (Sensebé and Bourin, 2008; Reinhardt et al., 2011; Sensebé et al., 2011). Consequently, many efforts have been made to replace animal derived supplements and reagents with human blood derivatives as autologous serum (AS) (Stute et al., 2004), pooled human AB serum (PhABS) (Bieback et al., 2009), platelet lysate (PL) (Hemeda et al., 2014) or a combination of them (Kocaoemer et al., 2007; Muraglia et al., 2015).

In this view, during the last years our group has been focusing on the definition of a clinical grade protocol to culture bone marrow-derived multipotent cells by replacing FBS

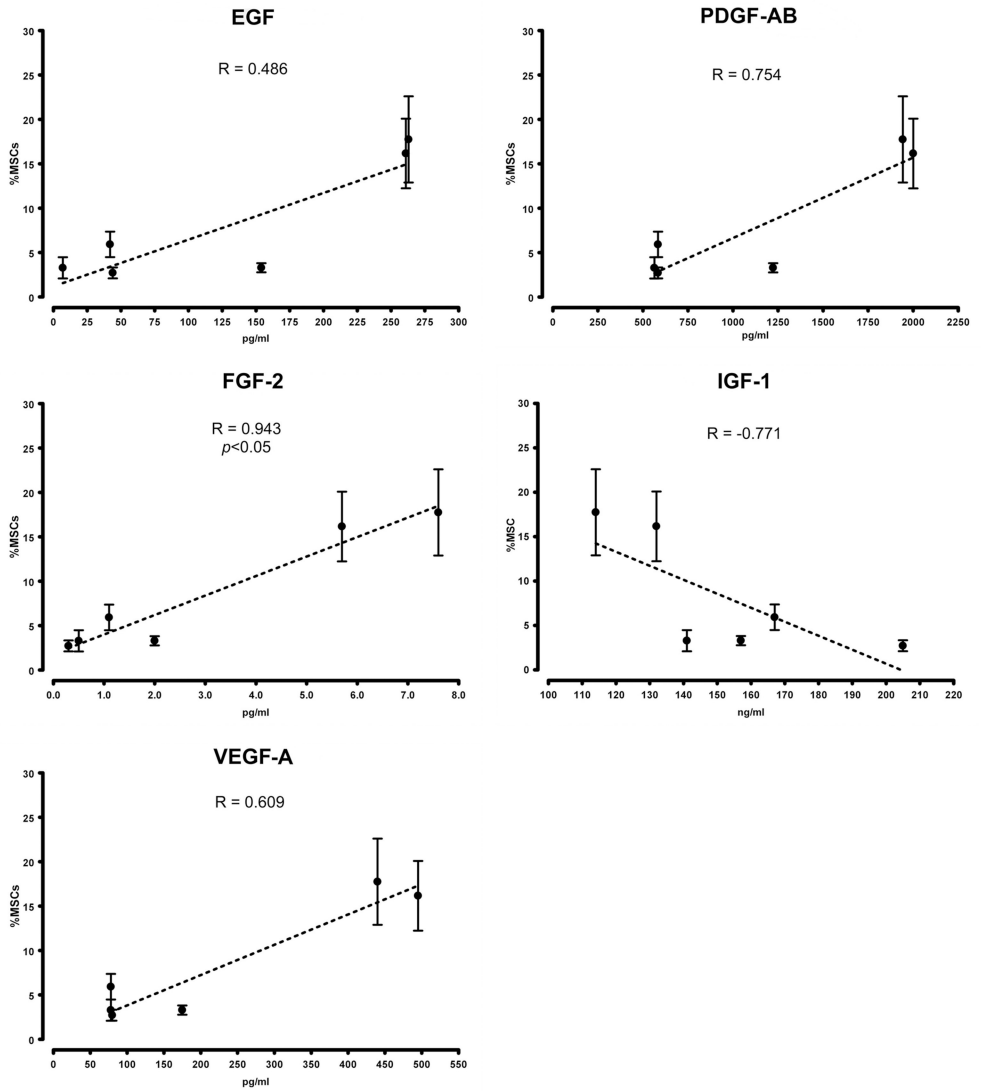

FIGURE 3 | Growth factor serum content correlated with cell composition in primary cultures. Higher concentrations of EGF, FGF-2, PDGF-AB, and VEGF-A were detected in PhABS that gave origin to cultures with higher percentages of "contaminating" MSC-like cells. Good performance in the initiation of MPC cultures at high grade of purity correlated with elevated levels of IGF-1.

with AS or PhABS. Our efforts led to the first description of *mesangiogenic progenitor cells* (MPCs) as previously not described cell population, co-isolated in AS-supplemented MSC cultures (Petrini et al., 2009). In parallel, a group of colleagues demonstrated the long-term efficacy and safety of this kind of cell products for the healing of atrophic pseudarthrosis of the upper limb (Giannotti et al., 2013). In this paper, Authors also hypothesized that MPCs detected in the applied cell populations, even if at low percentage (1–10%), could contribute to the long lasting healing (follow-up at 76 months) reported for the eight enrolled patients. This interesting hypothesis could be corroborated by the idea that implanted MPCs could provide efficient tissue regeneration, differentiating into *early* MSCs, as well as contribute to the vascularization of the engineered construct due to their demonstrated angiogenic properties (Fazzi et al., 2011). After these encouraging clinical results, we believe that manufacturing MPC-based medicinal products could provide a new promising tool in skeletal tissue engineering.

As MPCs have been reported being not-proliferating cells, the expansion is not actually permitted. Nonetheless, the frequency of MPCs has been estimated around 1% of hBM-MNCs, which is hundred to thousand times higher respect to MSCs (Trombi et al., 2009), leading us to hypothesized cell-based therapies involving this non-expanded multipotent progenitors. The possible application of not *in vitro* amplified cell products carries significant advantages in terms of: (i) a reduced risk of cell transformation, (ii) reduced cellular senescence, and (iii) reduced exposition to bacterial and viral contamination, minimizing the

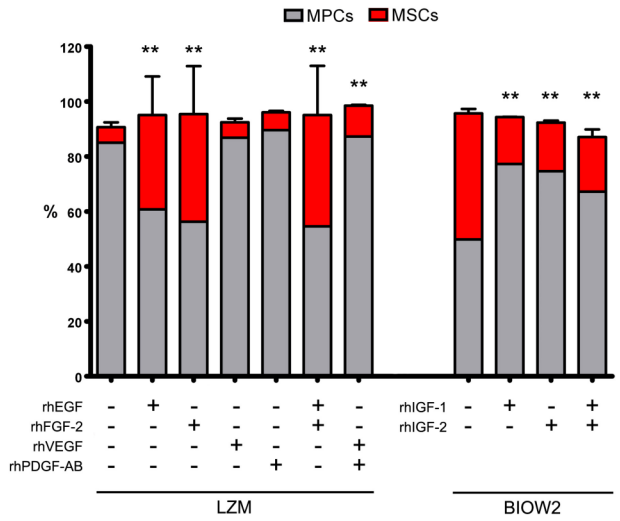

FIGURE 4 | Managing hGF medium contents, by adding recombinant factors, affected the sera performance during MPC isolation. Adding rhEGF or rhFGF-2, alone or in combination, to the cultures supplemented with good performance serum (LZM), resulted in significantly increased MSC-like population (red bars). Under these conditions, MPC/MSC ratio was comparable to those reported applying MSC-inducing PhABS. Treating with rhVEGF or rhPDGF-AB had no effects on MPC isolation, but a combination of those two hGFs produced mild but significant increase in MSC-like counterpart. BIOW2 batch was selected as PhABS with poor performance, evaluating the effects of rhIGF-1 and rhIGF-2 during MPC isolation. The treatment with rhIGFs, either alone or in combination, produced a 50% reduction of the MSC-like population (**$p < 0.01$, respect to control).

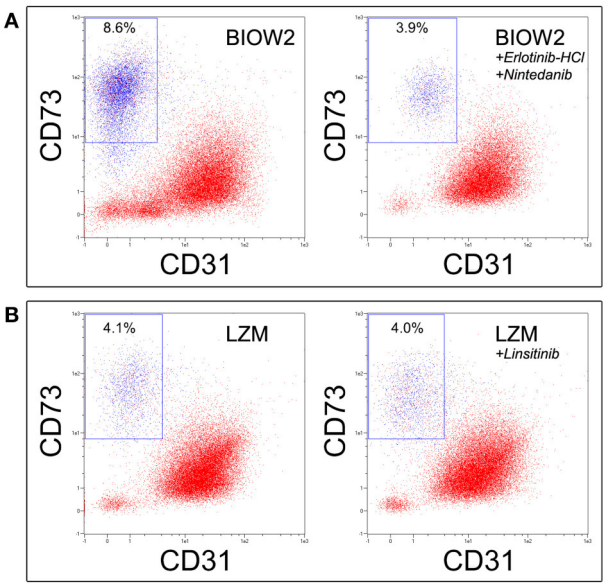

FIGURE 5 | *Erlotinib*-HCl in combination with *Nintedanib* allowed recovering quality MPC products applying sera with poor performance. (A) The simultaneous inhibition of EGFR, FGFRs, PDGFRs and VEGFRs during culture of hBM-MNCs in BIOW2 serum batch, produce a 50% reduction in the $CD73^{+}CD90^{+}$ population, leading to a cell product comparable to once produced in LZM. **(B)** Conversely, the inhibition of IGF-1R exerted by *Linsitinib* did not affect the MPC production.

culture time (Schneider et al., 2010). However, this could provide limited number of cell doses forcing to investigate any possible clinical application of MPCs in the context of personalized cell therapies, which involved small-scale CBMP production similar to that described by Giannotti et al. (2013). Anyway, in the attempt to define manufacturing conditions, selective for MPCs, we observed large variability in MPC yield using AS as well as different manufacturers and batches of commercial PhABS.

In general, CBMPs represent complex biological products displaying high rate of intrinsic variability, mainly derived from two sources: (i) process starting material and (ii) process conditions (Williams et al., 2012). In particular for MPC-based product, the first mentioned source of variability could not be eliminated in the autologous context where starting materials are obtained by different patients. Moreover, even if the variability related to the production process could be significantly reduced applying automated and well-controlled manufacturing process (Liu et al., 2010; Pacini, 2014), the serum supplementation represents an inescapable source of variability. Here we showed such variability to correlate with some of the most important growth factors contained in platelet lysates (Fekete et al., 2012), whose concentrations in the serum fluctuate regardless of donors' age, sex or production method. Best performance in MPC production was obtained with serum concentrations of IGF-1 over 150 ng/ml combined to low levels of EGF (<160 pg/ml), FGF-2 (<2.0 pg/ml), PDGF-AB (<1200 pg/ml), and VEGF-A (<180 pg/ml). Screening these five growth factors could provide a simple means to select high quality HS batches for the production of MPC-based CBMPs. Moreover, data presented here also provide interesting application for *Erlotinib*-HCl and *Nintedanib* in the production of MPC in autologous serum, where any possible source of variability, related to fluctuations in hGF serum concentration, could be mitigated by receptors inhibition. This could possibly results in qualified MPC products independently by patient sex, age, etc.

Taking in consideration only hGFs as serum factors influencing MPC cultures, represents the mayor limit of this study. Serum is highly complex biological fluid composed also by many other macromolecules (i.e., cytokines and immunoglobulins), different lipids, hormones and other small molecules. Further investigations are needed to exhaustively define the essential components required to produce high quality MPC-based cell product. Anyway, here we clearly demonstrate that managing the interactions of these hGFs with their receptors could also represents a new tool in MSC production. In fact, culturing bone marrow in AS, or PhABS, and in presence of *Erlotinib*-HCl and *Nintedanib* could lead to highly purified MPCs that could act as culture initiating cells to produce more homogeneous and synchronized MSC cultures. Those cultures could be easily obtained applying the same HS, by removing receptor inhibition and supplementing with recombinant hGF cocktail including at least EGF and FGF-2, as demonstrated here. Our findings are in accordance with Yamaguchi et al. that reported comparable results between FBS and PhABS, in

MSC culture, when PhABS cultures had been supplemented with 10 ng/ml of FGF-2 (Yamaguchi et al., 2002). However, the extensive literature on serum supplements in MSC cultures is very controversial. A number of authors showed no significant differences between AS and FBS (Yamamoto et al., 2003; Spees et al., 2004; Stute et al., 2004), while Shigeno and Ashton obtained greater response using AS (Shigeno and Ashton, 1995), in contrast to the extensively reported higher performances applying FBS (Koller et al., 1998; Kuznetsov et al., 2000). It is reasonable hypothesized that the origins of those controversial data, in MSC manufacturing, reside in the lack of preliminary characterization of hGF content in the HS applied. We also believe that those inconsistencies originate from restricting focus on MSCs, without taking into account the positive effects of the MPC presence within the CBMP once implanted *in vivo*.

HS batches rich in EGF and FGF-2 apparently worsen the production of standard monomorphic MPC cultures probably supporting the proliferation of MSC-like cells from other distinct and rare progenitors reported in the bone marrow. These mesengenic progenitors could include skeletal stem cells (SSCs), recently described as the genuine skeletal tissue stem cells (Bianco and Robey, 2015) or CD146-positive non-stem osteoprogenitor (Sacchetti et al., 2007). This hypothesis is validated by our data on growth factor receptor expression. Significantly higher levels of *EGFR*, *FGFR2,* and *PDGFRA* were reported in MSCs suggesting that these specific growth factors could have trophic effect on MSC-like cells (Ng et al., 2008) instead of inducing MPCs to differentiate. Conversely, VEGF-A was detected at higher concentrations in MSC-inducing sera while *KDR* was more expressed in MPCs, together with *TGFBR2*, suggesting a role for VEGF-A and possibly TGF-β in MPC differentiation toward the mesenchymal lineage. IGF-1 appeared to be acting as MPC-promoting factor as its levels were particularly high in MPC-inducing sera LZM, LZMF, and SERL, mirroring IGF receptor expression in MPCs.

In conclusion, we believe that MPCs represent also a promising alternative to hBM-MNCs as culture-initiating cells in the production of clinical grade MSCs. A purified and well-characterized progenitor cell population cultured under specific controlled conditions would significantly improve CBMP reproducibility and consequently the predictability of pre-clinical studies. MPCs are found at frequencies one to two logs higher than the other MSC progenitors described in bone marrow. Moreover, future clinical applications for CBMPs based on undifferentiated and not expanded MPCs could take advantage from their angiogenic potential that is suggestive of possible beneficial effects on neo- or re-vascularization of target tissues. A complete definition of the active growth factor cocktail for MPC efficient isolation, expansion, and differentiation is still required also in order to possibly develop specific chemically defined media (CDM), which will eliminate the biological variability related to the serum supplementation.

AUTHOR CONTRIBUTIONS

MM: Conception and design, Data collection, assembly, analysis, and interpretation. SB: Data collection, assembly, analysis, and interpretation. VC: Collection and assembly of RT-PCR data. FF: Collection and assembly of ELISA assay data. FP: Data collection. IP: Conception and design. SP: Conception and design; Data collection, assembly, analysis, and interpretation; Manuscript writing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcell.2016.00114

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Are MSCs angiogenic cells? New insights on human nestin-positive bone marrow-derived multipotent cells

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Recent investigations have made considerable progress in the understanding of tissue regeneration driven by mesenchymal stromal cells (MSCs). Data indicate the anatomical location of MSC as residing in the "perivascular" space of blood vessels dispersed across the whole body. This histological localization suggests that MSCs contribute to the formation of new blood vessels *in vivo*. Indeed, MSCs can release angiogenic factors and protease to facilitate blood vessel formation and *in vitro* are able to promote/support angiogenesis. However, the direct differentiation of MCSs into endothelial cells is still matter of debate. Most of the conflicting data might arise from the presence of multiple subtypes of cells with heterogeneous morpho functional features within the MSC cultures. According to this scenario, we hypothesize that the presence of the recently described Mesodermal Progenitor Cells (MPCs) within the MSCs cultures is responsible for their variable angiogenic potential. Indeed, MPCs are Nestin-positive CD31-positive cells exhibiting angiogenic potential that differentiate in MSC upon proper stimuli. The ISCT criteria do not account for the presence of MPC within MSC culture generating confusion in the interpretation of MSC angiogenic potential. In conclusion, the discovery of MPC gives new insight in defining MSC ancestors in human bone marrow, and indicates the *tunica intima* as a further, and previously overlooked, possible additional source of MSC.

Keywords: mesenchymal stromal cells, endothelial differentiation, angiogenesis, adult stem cells, bone marrow, nestin, neo-vascolarization, *in vivo* MSC

DISCOVER, ISOLATION, AND CHARACTERIZATION OF MSCs

In the late sixties, A. J. Friedenstein and coworkers first described the multipotent mesenchymal stromal cells (MSCs). When human bone marrow (BM) cells were cultured in plastic dishes colonies of adhered fribroblastoid cells proliferate and hematopoietic precursors progressively disappear. These cells have been named C*olony Forming Units of Fibroblastoid cells* (CFU-Fs) because of their ability to form large colonies on plastic surfaces. MSCs are able to differentiate into chondrocytes and osteoblasts, *in vitro* (Friedenstein et al., 1968), and *in vivo* (Friedenstein et al., 1974). By that time, T. M. Dexter and colleagues developed a culture system to study hematopoiesis and demonstrated that bone marrow hematopoietic stem cells (HSCs) were unable to adhere onto the culture flasks but necessitate an underlying layer of adherent cells that mimics the bone marrow stromal compartment (Dexter et al., 1977). After the demonstration that CFU-Fs originate from the bone marrow stroma, their name was changed in bone marrow stromal cells (Lanotte et al., 1981). In 1991, A. I. Caplan suggested the presence of a stem cell population in the adult BM able to differentiate into multiple mature cell lineages sharing a common precursor in the mesodermal layer of the embryo. Therefore, these cells were named "mesenchymal stem cells" (Caplan, 1991). Subsequently, the differentiation potential of MSCs into multiple mature lineages has been confirmed: these cells have a stable phenotype that can be easily expanded in culture and maintain the ability to differentiate into osteoblasts, chondrocytes, adipocytes, tenocytes, myocytes and stromal cells supporting hematopoiesis (Pittenger et al., 1999). MSCs became popular when K. Le Blanc et al. showed that MSCs are "invisible" to the immune system because they express only the class I Major Histocompatibility Complex (MHC-I) but not the class II and co-stimulatory molecules such as CD40, CD80, and CD86. This is relevant in the prospective of allogeneic transplantation with possible therapeutic applications (Le Blanc et al., 2003).

The isolation and the expansion of MSCs are easily feasible from the adult bone marrow in most cellular laboratories. Therefore, MSCs have been largely evaluated and employed in several premature clinical trials. However, a precise characterization of these cells is still missing as well as standardized protocols for their isolation and expansion. Consequently, results are controversial and the biology of MSCs is still unclear.

More recently, the International Society for Cellular Therapy (ISCT) has proposed minimal criteria to define MSCs (Dominici et al., 2006):

(1) MSCs are plastic-adherent cells in standard culture conditions,
(2) MSCs express the surface marker: CD105, CD73, and CD90 and do not to express the hematopoietic markers: CD45, CD34, CD14, and CD11b, and
(3) MSCs must be able to differentiate toward osteogenic, adipogenic and chondrogenic lineages when exposed to the proper conditions *in vitro*.

Finally, MSCs have been re-named "mesenchymal stromal cells" modifying the term "stem" into "stromal" in order to maintain the same acronym and avoid possible over-interpretation of their limited pluripotent potential (Horwitz et al., 2005).

TISSUE ORIGINS AND DISTRIBUTION OF MSCs

Cells matching the ISCT criteria can be isolated not only from the bone marrow but also from other adult and the fetal tissues (De Bari et al., 2001; Zuk et al., 2001; In't Anker et al., 2004; Seo et al., 2004). In these studies the culture condition adopted to establish BM-derived MSCs were applied to cells isolated from other tissues, in order to determine if MSCs reside also in different organs. In mice, L. da Silva Meirelles et al. demonstrated that long-term culture of MSCs could be established from a wide range of different adult tissues including fat, muscles, pancreas, vena cava, kidney glomerulus, aorta, brain and many others alongside bone marrow (da Silva Meirelles et al., 2006). Notably, all the cell populations that L. da Silva Meirelles established, independently from their origin, were long-term culture of adherent cells, with MSC phenotype and able to differentiate into mesenchymal cell lineages. These data suggest the presence of MSCs in virtually in all organs and tissues of the body. Three hypotheses try to explain the tissue distribution of MSCs:

(1) MSCs permanently reside in multiple tissues and organs,
(2) MSCs reside in only in particular tissues but can circulate in blood, and
(3) MSCs are circulating blood cells.

The presence of CFU-Fs in blood of adult mammals was shown in 2007 (He et al., 2007). However, the contamination with fragments of connective tissue could not be ruled out to justify the presence of MSCs in the collected sample. Disputes remain regarding the existence of circulating MSCs (Roufosse et al., 2004; Kuznetsov et al., 2007). L. da Silva Meirelles demonstrated the presence of MSCs within tissue cleaning the organs using intravascular perfusion before their collection. Nonetheless, the possibility that MSCs may circulate locally or systemically under non-physiological conditions, i.e., tissue injury, is not excluded. Although, the features of MSC from different organs are similar, mild differences in differentiation potential and surface markers have been reported. These differences have been related to the influence of a modified local environment (niche) present in different site of the body.

MSC can be isolated from the wall of blood vessels (Doherty et al., 1998; Bianco et al., 2001), and in 2007, B. Sacchetti et al. demonstrated a common phenotype for BM-derived CFU-Fs and Adventitial Reticular cells (ARCs). ARCs populate sinusoids and lay in close contact with the endothelium. Strong evidence indicate that the fibroblastoid colonies described by Friedenstein *in vitro* originate from the ARCs isolated *ex vivo* (Sacchetti et al., 2007). Stromal progenitors in human BM that reside in the sub-endothelial layer of sinusoids strongly express the melanoma-associated adhesion molecule (MCAM/CD146). A. Tormin et al. confirmed that CD271 is an *in vivo* marker of BM-derived MSCs (Quirici et al., 2002) and described a subset of CD271+ cells that express CD146. Cells expressing CD271 and CD146 are the ARCs present in the sub-endothelial layer of sinusoids. The remaining CD271+/CD146− cells maintain the MSC features but *in vivo* reside in the trabecular bone-lining endosteal niche (Tormin et al., 2011). Therefore, there are at least two cells able to generate MSCs from the bone marrow one in the perivascular (CD271+/CD146+) and one in the endosteal niche (CD271+/CD146−).

M. Corselli et al. isolated two distinct MSC progenitors from the stroma vascular fraction (SVF) of the adipose tissues (Corselli et al., 2012). CD34−CD146+ pericytes encircling capillaries and microvessels and CD34+CD146− adventitial cells surrounding larger arteries and veins. MSC-like cultures can be expanded from both these populations, suggesting a vascular origin for the MSCs of the adipose tissue, similarly to what observed in bone marrow. A hierarchical organization of cell differentiation has been proposed for the vascular progenitor of MSCs being the adventitial cells the precursors of pericytes. Indeed, under proper conditions, ARCs differentiate into pericytes *in vitro*. Later, M. Crisan et al., isolating the cells through CD146, demonstrated the perivascular origin of MSCs in multiple organs (Crisan et al., 2008).

A new hypothesis that MSCs are localized *in vivo* in "perivascular" spaces that extend through the whole post-natal organism has been proposed. While this latest hypothesis is gaining consensus among researchers, the term "perivascular" is somehow ambiguous because it include the proximity of vessel and the wall itself. More precisely, two intra-vessel wall compartments, the *adventitia* and *sub-endothelium*, have been indicated as possible locations for these two MSC progenitors. The relationship between these two progenitors remains obscure, even if they have been largely characterized. Their histological localization, suggests a role of MSC in blood vessel formation *in vivo*. MSCs can directly differentiate into vascular cells (endothelial cells and smooth muscle cells) and/or as supporting vascular (re)-generation in response to the paracrine secretion of stimulating factors (Lin and Lue, 2013).

CONTROVERSIES ABOUT THE ANGIOGENIC POTENTIAL OF MSCs

One of the most interesting debates regarding MSCs concerns their angiogenic potential. Due to the possible role of MSC in therapeutic (re)-vascularization, an increasing number of studies *in vitro* and *in vivo* have been performed (reviewed in Vittorio et al., 2013).

The formation of new blood vessel can be divided in:

(1) Vasculogenesis: *de novo* formation of blood vessels from the endothelial precursors or angioblasts,
(2) Angiogenesis: includes sprouting of existing vessels and intussusceptive angiogenesis, and
(3) Arteriogenesis: remodels a pre-existing collateral circulation (Makanya et al., 2009; van Royen et al., 2009; Melero-Martin et al., 2010; Carmeliet and Jain, 2011; Potente et al., 2011).

Although MSCs support these processes through the release of angiogenic factors and protease (reviewed in Watt et al., 2013), the relevance of their differentiation into endothelial lineages is still debated.

The demonstration of MSC commitment toward endothelial lineage is often limited to the detection of the upregulation of typical EC surface molecules including CD31, CD34, VEGF receptors (VEGFR1, VEGFR2) and von Willebrand factor (vWF). As phenotype modification is insufficient to demonstrate differentiation, additional functional tests are often performed. These tests include *in vitro* tube formation on Matrigel® and uptake of acetylated-low density lipoproteins (Ac-LDL). Nonetheless, it might be inaccurate describing these differentiated MSCs as fully mature and functional ECs basing on these *in vitro* assays. For example, Ac-LDL uptake has been described also in macrophages and pericytes (Voyta et al., 1984).

A large effort was spent for the optimization of protocols able to induce endothelial differentiation of MSCs. VEGF stimulates the differentiation of MSCs into ECs. In 2004, J. Oswald et al. demonstrated that confluent human BM-derived MSCs cultured in 2% fetal calf serum (FCS) and 50 ng/ml VEGF for a week, displayed upregulation of endothelial surface markers including VEGFR1, VEGFR2, VE-Cadherin, VCAM-1, and vWF. Moreover, when incubated on Matrigel® *in vitro*, MSCs formed characteristic capillary-like structures (CLS) (Oswald et al., 2004). Similarly, M. Jazayeri et al. cultured human BM-derived MSCs in medium supplemented with 5% FCS, IGF and VEGF and detected CD31, vWF, Tie2, VCAM1, and VE-cadherin on the cell surface. In addiction, applying electron microscopy Authors showed the presence of typical EC morphological features including Weibel-Palade bodies, tight junctions and caveolae (Jazayeri et al., 2008). Similar results were also achieved using "endothelial growth medium-2" (EGM-2, which contains VEGF, EGF, FGF-2, IGF-1, hydrocortisone, heparin, ascorbic acid and 2% FCS) (Liu et al., 2007) and MSCs isolated from the adipose tissue (Cao et al., 2005; Fischer et al., 2009). Conversely, V.D. Roobrouck et al. reported that VEGF treatment of human BM-derived MSCs significantly increased mRNA expression of CD34, VEGFR1, and VEGFR2, but not of Tie-2 and vWF or CD31 that was even decreased. These MSCs also failed forming CLS in Matrigel® assays, (Roobrouck et al., 2011). In parallel, W. Fan et al. demonstrated that human BM-derived MSCs cultured in the presence of different concentrations of VEGF did not show increase in CD31, vWF or VEGFR2 expression (Fan et al., 2011).

In vivo G. V. Silva et al. demonstrated that MSCs applied in a region of myocardial ischemia can differentiate into smooth muscle cells and endothelial cells leading to increased vessel density and an improvement the cardiac function, in a canine model (Silva et al., 2005). Nonetheless, there is a consolidating concept that the angiogenic effect of MSCs is predominantly caused by their paracrine actions rather than their EC trans-differentiation potential. A. Al-Khaldi et al. showed that, in the murine Matrigel® plug assay, more than 99% of the new-formed blood vessels originated from host-derived EC, while a small portion of injected BM-derived MSCs were found in the close proximity of- or within blood vessels (Al-Khaldi et al., 2003). Moreover, the observation that MSCs *in vitro* committed through endothelial lineages were not superior to "*naïve*" MSCs in stimulating *in vivo* angiogenesis, may underline the relevance of the secretion of pro-angiogenic factors that is also sustained by uncommitted MSCs (Liu et al., 2007; Fan et al., 2011). Thus, the up-regulation of endothelial marker, under specific culture conditions, could represent an *in vitro* artifact and not a real differentiation into functional endothelial cells. BM-derived MSCs seems to be an important regulator of neo-vascularization by the secretion of pro-angiogenic factors as well as by differentiating into functional pericytes able to stabilize the new-formed vasculature (Au et al., 2008), rather than a source of endothelial progenitor cell.

Most of the controversial data about endothelial differentiation of MSCs need to be discussed as consequence of the sub-optimal protocols of differentiation (Janeczek Portalska et al., 2012). Moreover, a critical issue is the heterogeneity of the primary MSC cultures used to generate endothelial progenitors.

HETEROGENEITY OF CULTURE EXPANDED MSCs

The anatomic localization and the physiological function of MSCs are not clearly characterized. MSCs are commonly isolated from long-term cultures; therefore, it remains difficult to determine the primary cells of origin. The loose ISCT criteria hamper the identification of unique precursors of MSCs. Indeed, several types of primary cells with different features can fulfill the definition of MSCs *in vitro*. Being the definition permissive, the presence of a unique common precursor for cells with MSC features cannot be hypothesized. In BM, MSCs can originate from both perivascular and endosteal progenitors, therefore, it is difficult to distinguish if there is a unique common precursor or if the loose ISCT definition is unable to identifies two different progenitor populations. However the clinical applications of MSCs are only partially limited by the incomplete characterization of the progenitor cells.

The heterogeneity of MSC cultures, defined according to ISCT criteria, is becoming evident in more recent articles and brings into question the utility of these ambiguous criteria. From the beginning, different terms have described the morphology of plastic-adherent cells: fibroblastoid (Werts et al., 1980), giant fat cells and blanket cells (Allen and Dexter, 1983), spindle shaped flattened cells (Kuznetsov et al., 1997) and very small round cells (Colter et al., 2001). Thus, mesenchymal cell morphology seems to be highly dependent on culture conditions: supplements, seeding density, number of passages and culture time (Wagner and Ho, 2007; Barachini et al., 2009). It is still unclear if there is any relation between these different morphology and cell functions.

There is not a consensus on the surface markers of MSCs, aside from the unspecific CD105, CD90, CD44, and CD73, because different laboratories use different sets of antigens. Therefore, the differentiation into mature cells with a mesengenic ancestor seems to be the more reliable and stringent criteria to define MSCs. However, the differentiation potential of MSC is variable. This variability is observed between different donors (Phinney et al., 1999) and also within different colonies obtained from the same subject (Russell et al., 2010). Indeed, colonies obtained from the same individual could be characterized as mono-, bi- or tri-potent on the basis of their ability to differentiate into, respectively one, two or three types of tissue: osteogenic, chondrogenic and adipogenic lineages. Moreover, it has been clearly demonstrated that repeated passages progressively reduce the multi-lineage differentiation ability, introducing a further element of complexity (Muraglia et al., 2000). It is possible to

hypothesize that the angiogenic potential of MSC is subject to a similar variability that is influenced by the same factors.

MSCs are heterogeneous not only among different colonies but also within the same colony (Digirolamo et al., 1999). Cells show variable differentiation potential in relation to their topographic localization inside the colony. Cells from the center and the margins of the colony differ for shape, differentiation potential and surface markers (Ylöstalo et al., 2008; Sengers et al., 2010). Therefore, the term "multipotent mesenchymal stromal cells" does not identify a population of cells with uniform features and unambiguous potential but refers to a highly heterogeneous population that is dramatically affected by donors characteristics (Russell et al., 2013), isolation methods (Wagner and Ho, 2007; Barachini et al., 2009), culture conditions (Bieback et al., 2009).

Several possible mechanisms may explain the basis of the MSC heterogeneity, beside the already described variability introduced by *ex vivo* procedures (Pevsner-Fischer et al., 2011). Hypotheses on the origin of this variability include: stochastic events, occurring during expansion and differentiation and a possible *in vivo* heterogeneity of the isolated cell populations. In this latter hypothesis, specific culture conditions select, or simply promote, particular subpopulations of MSCs giving reason of the observed heterogeneity and the morpho-functional variability. According to this scenario, numerous multipotent cell populations can be described in bone marrow, some of them able to differentiate into lineage from the three germ layers: endoderm, mesoderm and ectoderm (triploblastic differentiation). However, the isolation and successive characterization of these cells is strictly dependent on the application of specific culture conditions. For example, "marrow-isolated adult multipotent inducible (MIAMI)" cells can differentiate into, neural and pancreatic-like cells in addition to skeletal tissue lineage (D'Ippolito et al., 2004). The isolation and expansion of MIAMI cells require specific culture conditions with low oxygen tension.

Recently a unique multipotent sub-population in adult human BM-derived MSCs has been isolated using fluorescent activated cell sorting (FACS) for stage-specific embryonic antigen 3 (SSEA-3). BM-derived MSCs show poluripotency-differentiation properties (Kuroda et al., 2013). Interestingly, stress conditions could enrich the expression of SSEA-3 in cultured MSCs (Kuroda et al., 2010). Y. Kuroda et al. demonstrated that long-term trypsin incubation could increase the recovery of cell clusters containing pluripotency-associated markers and renamed these cells as "multi-lineage differentiating stress-enduring" (MUSE) cells.

Thus, if mild modifications in the culture conditions, or in the culture procedures, can induce/preserve an embryonic-like differentiation potential in BM-derived cells, it is reasonable to suppose that angiogenic potential behaves similarly and is significantly affected by manipulation *in vitro*.

MESODERMAL PROGENITOR CELLS (MPCs) IN ADULT HUMAN BONE MARROW

In 2007, we attempted to optimize MSC culture conditions for clinical application selecting media without supplements of animal origin. When medium was supplemented with autologous serum instead of that of bovine origin, a small population of cells with distinct shape was noticed. These cells presented rounded, fried-egg shape, instead of the usual spindle morphology of MSCs, were highly refractive and remain firmly attached to the plastic during trypsin digestion

In 2008, by replacing fetal bovine serum (FBS) with pooled human AB serum (PhABS) in the culture medium of human BM cells, we were able to characterize this new population of adherent cells (Petrini et al., 2009). These cells are quiescent: Ki-67 negative; with long telomeres and express the pluripotency-associated transcription factors Oct-4 and Nanog instead of RUNX2 and Sox9 typical for MSC-phenotype (Pacini et al., 2010). Phenotypically, these cells share the expression of CD105 with MSCs but lacked expression of CD73, CD90, CD166, CD271 and those other markers typical of the mesenchymal phenotype such as MSCA-1. Interestingly, this cell population rapidly produces mesenchymal offspring when supplemented with FBS or human cord blood serum. Thus, this novel population of cells, isolated from the BM, has *in vitro* characteristics of a progenitor of the mesengenic lineage and therefore has been named "*Mesodermal Progenitor Cells*" (MPCs).

ISOLATION OF MPCs FROM HUMAN BM SAMPLES

Method for the isolation of MPCs from BM samples is feasible, inexpensive and based on selective culture conditions (Trombi et al., 2009). Initially, MPCs were co-isolated together with MSCs applying media supplemented with autologous serum or PhABS, in culture. It became evident that MPCs have different adhesion properties compared to MSCs. In fact, applying standard trypsin-based cell detaching protocols, MSCs were entirely harvested while most of the MPCs remain firmly attached to the plastic surface and required different proteases' solution (TrypLE Select® from LifeTechnologies) to be detached. Therefore, plastic features and coating of the culture surfaces influence the proportion of MPCs and MSCs in the primary cultures. We firstly tested not gas-treated hydrophobic plastics, usually applied for cultures in suspension, and surprisingly MPCs were able to attached on that surface also. Conversely, the hydrophobic conditions resulted not permissive for MSC. Thus, a selective culture could be established using these conditions allowing the recovery of MPCs with a purity of more than 95%. We also noticed that a higher yield of MPC recovery was achieved using higher seeding densities, than that usually applied for MSC isolation. In summary, PhABS supplementation and high density seeding on hydrophobic plastics were the selective culture conditions necessary for MPCs isolation from BM-MNCs. This method has been consolidated and it is highly reproducible allowing the quality screening of the MPC preparations before their employment in the different studies.

The mechanisms behind the difference, in MPC recovery, between culturing cells in FBS or PhABS-containing medium are still unknown. Nonetheless, we demonstrated that the addition of PhABS, even in small percentages, to FBS primary cultures allows MPC isolation, suggesting the presence of undetected agents able to induce MPCs in the human serum (Trombi et al., 2009). Conversely, when FBS is added to cultures grown in PhABS, cells differentiate into MSCs without any significant reduction in the number of MPCs indicating a possible semi-conservative proliferation of MPCs. What characterized FBS against PhABS is the different origin in terms of species (bovine instead of human) and

stage of body development (fetal against adult). The differentiation of MPCs into MSCs is induced replacing PhABS with FBS (Petrini et al., 2009), or using human cord blood-derived serum (unpublished data). These preliminary results suggest that media supplementation with fetal sera represent the culture condition for the mesengenic induction of MPCs, independently from the adopted species.

Mesengenic potential of MPCs

Later, we have been able to demonstrate a hierarchical multistep model of mesenchymal differentiation with at least three different populations of multi-potent cells (Fazzi et al., 2011). Indeed, MPCs can generate exponentially growing MSC cultures after 2 weeks of stimulation with differentiating conditions. The differentiation proceeds through the commitment into an intermediate cell population; we named *early* MSCs. Timing of MPC mesengenic differentiation was definitively clarified and specific morphologies, phenotypes and growing features of the three protagonists described (**Figure 1A**). Studying Wnt signaling activation during MPC differentiation, we showed that non-canonical Wnt5/Calmodulin pathway was involved in the commitment of MPCs into *early* MSCs and demonstrated that Calmidazolium Chloride, a Calmodulin inhibitor, was able to interfere with the differentiation only at this initial step while has no effect on passage from *early* to *late* MSCs.

Angiogenic potential of bone marrow-derived MPCs

From the beginning it has been clear that MPCs have angiogenic potential because they form capillary-like structures (CLS) after a multiple steps of differentiation (Petrini et al., 2009; Trombi et al., 2009; Pacini et al., 2010). Interestingly, the inhibition of the Wnt5/Calmodulin signaling pathway has no effects on the MPC differentiation toward endothelial lineage suggesting that Wnt signaling pathway activation finely regulates MPC fate. On the contrary of mesengenic, all the passage of angiogienic differentiation have not been described to date due to the lack of specific culture protocols. Indeed, to partially induce endothelial differentiation we applied protocols optimized for endothelial progenitor cells (EPCs) with mild modifications (Hill et al., 2003). This was sufficient to demonstrate the angiogenic potential of MPCs and suggested that exist a BM-derived endothelial progenitor cell distinct from EPCs.

The high expression of CD31 and Nestin in MPCs suggest the existence of a primitive progenitor for the endothelial lineages (Petrini et al., 2009; Trombi et al., 2009; Pacini et al., 2010). Nestin is a class VI intermediate filament protein originally described as a marker of neural stem cells that is expressed during the development of central nervous system (CNS) (Lendahl et al., 1990). Although Nestin expression is down regulated during the differentiation into neurons or glial cells (Dahlstrand et al., 1995), it can be detected in adult neural progenitor cells (Reynolds et al., 1992; Morshead et al., 1994) and in some CNS tumors (Tohyama et al., 1992). Frequently, Nestin is not expressed by the cancer cells themselves but can be observed in the endothelial cells of the tumor regardless of malignancy grade or its histotype (Sugawara et al., 2002). This suggests that Nestin can be a marker of proliferating tumor endothelial cells and not only of neuroepithelial elements. Therefore, Nestin expression correlates with angiogenesis because it is expressed in proliferating vascular endothelial cells of the tumor (Kim et al., 2002; Teranishi et al., 2007; Gravdal et al., 2009; Eaton et al., 2010). K. Sugawara et al. demonstrated a high expression of Nestin in bovine aortic proliferating endothelial cells in static culture. This expression rapidly decreases under conditions of laminar shear stress flow, suggesting that Nestin expression is typical of early proliferating endothelial precursors but is loss in mature endothelial cells of normal tissues. In the recent years, the emerging concept of Nestin as a novel early angiogenic marker is gaining consensus in normal ad tumor angiogenesis (reviewed in Matsuda, 2013).

MPCs express CD31/PECAM (Pacini et al., 2013) but rapidly loose these markers during differentiation contextually with the lost of Nestin (**Figure 1A**). Preliminary data show that MPCs can make "sprouting" when directly seeded in Matrigel® 3D-cultures but are not able to efficiently form CLS without a step of predifferentiation. Therefore, MPCs could represent a very staminal progenitor with angiogenic potential more immature than what is reputed to date.

MPCs DO NOT SHOW FEATURES OF PERICYTES OR ADVENTITIAL PROGENITORS

Pericytes surround blood capillaries, precapillary arterioles, postcapillary venules and collecting venules (Sims, 1986; Allt and Lawrenson, 2001); where they can be identify by the expression of CD146 together with less specific markers such as α-smooth muscle actin (α-SMA), desmin, NG-2, platelet-derived growth factor receptor (PDGFR)-α, aminopeptidase A and N, RGS5, and the promotertrap transgene *XlacZ4* (Gerhardt and Betsholtz, 2003).

Pericytes and Adventitial Progenitor Cells (APCs) belong to the same cell lineage, according to several authors (Tormin et al., 2011; Corselli et al., 2012). Pericytes and APCs differ in their position in the vessel wall morphology, and some surface markers. However, this distinction is not absolute because exist a continuum between the phenotype of the classical APCs and the typical pericytes when they are distributed along small vessels such as arteriole, capillary, and venule. It has been suggested that pericytes may reside under the endothelium even in large vessels and support the endothelial removal and repair after injuries. From this point of view, these cells can be considered a reservoir of MSC-like undifferentiated cells (da Silva Meirelles et al., 2006). Conversely, Corselli's data demonstrated that APCs differentiate into pericytes *in vitro*, suggesting a "centripetal" (from the *adventitia* toward sub-endothelial layer) relationship between these cells (Corselli et al., 2012). Both the "centrifugal" and the "centripetal" model assumed that the *intima* would not be involved in the generation of MSC-like cells.

Interestingly, our MPCs express the early angiogenic markers Nestin and CD31/PECAM, suggesting their plausible location in the *tunica intima*. Although the localization of MPCs in the lumen-facing wall of vessels has been not definitively demonstrated, preliminary histological evaluations revealed the expression of Nestin in the sinusoids, arterioles/venules and larger

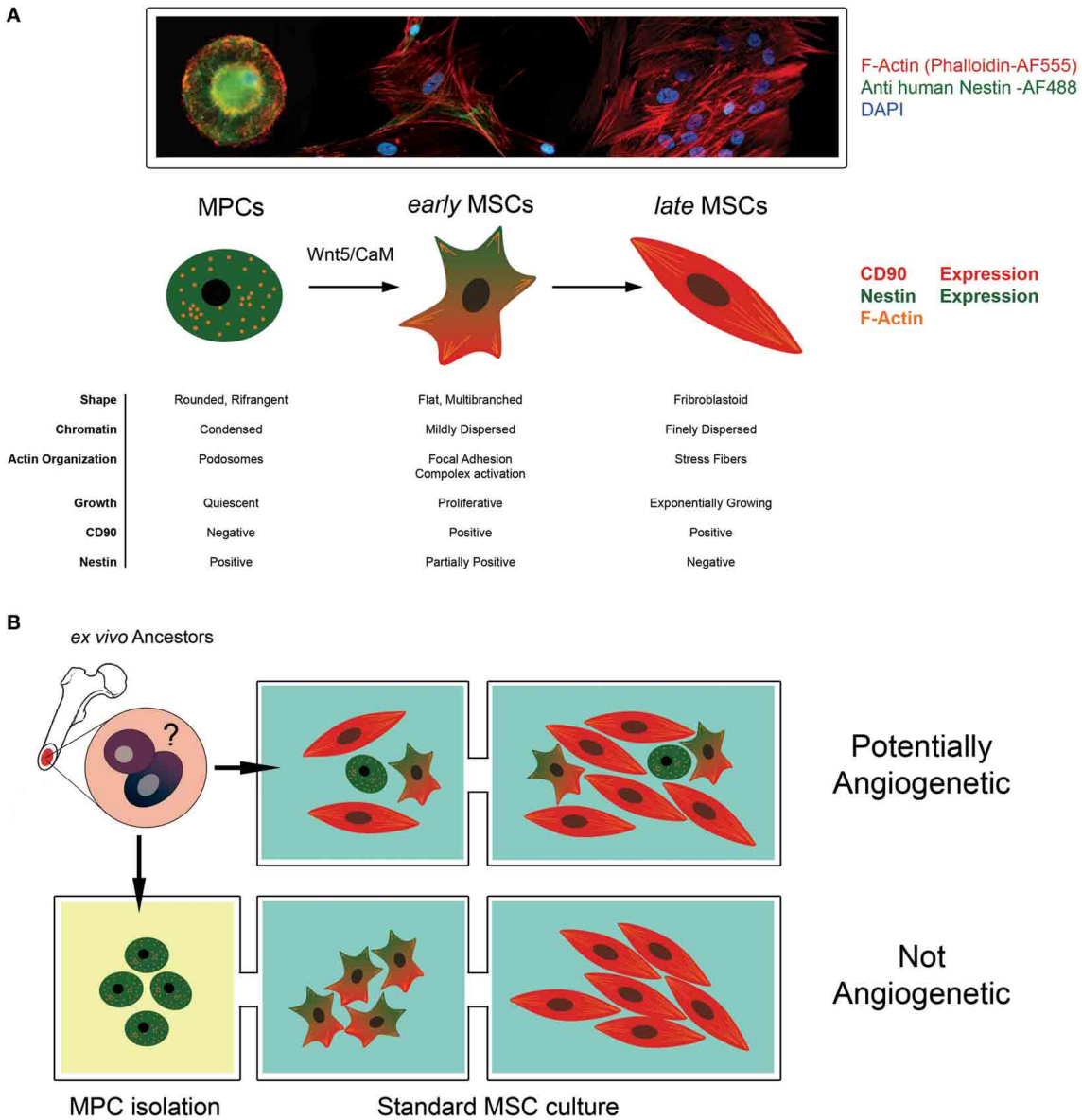

FIGURE 1 | The angiogenic potential of MSC cultures is controversial. **(A)** Mesodermal Progenitor Cells (MPCs) are CD90-, Nestin+ progenitors of MSCs. These cells remain in a quiescent state presenting a typical fried egg-shape, condensed chromatin and podosomal structures. When mesengenic differentiation is induced, MPCs differentiate into "*early* MSCs." *Early* MSCs slowly proliferate, express Nestin and CD90 (a MSC marker) and modify their shape because of Actin re-organization in focal adhesion complexes. Under persistent stimulation toward mesengenic differentiation, "*early* MSC" become "*late* MSC" showing exponentially growth, fibroblastoid shape and the ability to differentiate into skeletal tissues (fat, bone and cartilage). **(B)** According to this hierarchical model, MPCs can be considered a putative progenitor of the mesenchymal lineage *in vivo*, being present in the bone marrow mononuclear fraction. When supplemented with media containing FBS, these cells rapidly differentiate toward the mesengenic lineage and form asynchronous and heterogeneous cultures fulfilling the ISCT MSC criteria of definition. The undetected and unpredictable presence of sub-populations of MPCs, in culture could explain the variable angiogenic potential described for MSC in the literature. Conversely, the isolation of MPC and their subsequent differentiation using optimized protocol can allow the generation of synchronized and homogeneous mesenchymal stromal cells with a reproducible angiogenic potential.

vessels of bone marrow (**Figure 2**). A higher Nestin expression was observed in sinusoids' endothelium, whereas a lower Nestin expression was localized in only few cells located in the sub-endothelium and *adventitia*.

MPC do not express CD146, a specific markers of pericytes, as well as CD271. Therefore, it is unlikely MPCs reside in the *adventitia* or in the sub-endothelium (Petrini et al., 2009; Trombi et al., 2009; Pacini et al., 2010). Thus, any model of perivascular localization of MSC-like cells will be incomplete without the inclusion of the *tunica intima* among the possible sources of these cells.

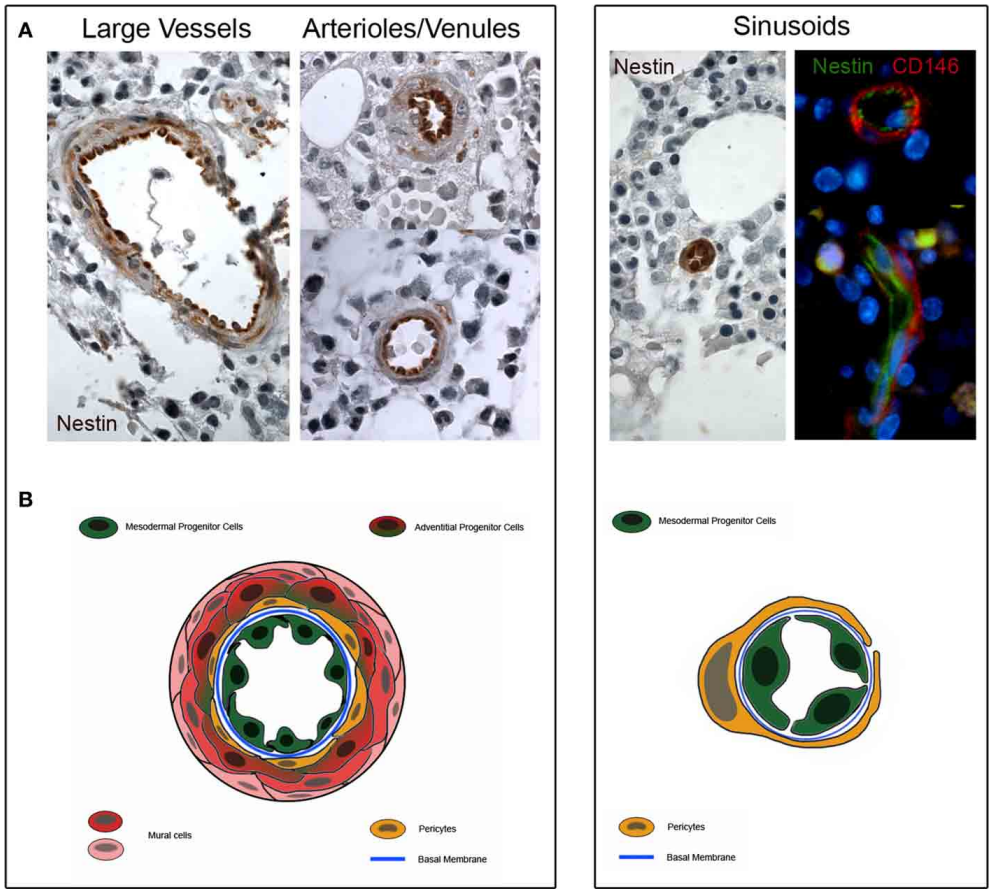

FIGURE 2 | Nestin expression in human bone marrow biopsies. (A) Consistent expression of Nestin is detectable in the *tunica intima* of large vessels, arterioles and venules (dark brown color). Few cells of the sub-endothelium and *adventitia* show a weakly positive Nestin staining (light brownish color). Furthermore, Nestin is highly expressed in the sinusoids and two colors immunofluorescence reveals that Nestin is positive in lumen-facing cells surrounded by CD146+/Nestin− pericytes. **(B)** Schematic representation of possible tissue distribution of MPC (represented in green) and other bone marrow vessels-residing cells.

NEW HYPOTHESIS ON ANGIOGENIC PROPERTIES OF BM-DERIVED MULTIPOTENT STROMAL CELLS

Recently, we have proposed that the yield of MPCs, co-isolated in the standard BM-MSC preparations, is influenced from the host, the batches of the serum and from the density of cell seeding (Petrini et al., 2009). The inter-population variability introduced by different donors and cell isolation protocols, affect the yield of MPCs, *early* MSCs or *late* MSCs. According to the hierarchical model, MPCs are progenitors of the mesenchymal lineage and account for 1–3% of mononuclear cells of the bone marrow (BM-MNCs). When BM-MNCs are seeded in standard FBS-containing media MPCs rapidly differentiate into mesengenic lineages forming the typical MSC culture in few days.

The variable angiogenic potential described in the literature of MSCs is probably related to the heterogeneous composition of the cultures expanded from the bone marrow that includes sub-populations of MPCs and MSCs when defined according to ISCT criteria (**Figure 1B**). Indeed, the expanded or exponentially growing MSCs are Nestin-negative and *de facto* coincide with our *late* MSCs. Late MSCs do not retain any angiogenic potential because these cells are already committed toward other mesengenic lineage (**Figure 1B**). Most of the reports do not apply specific protocols for MPC's isolation; thus, these MSC cultures represent an uncontrolled heterogeneity of multipotent cells with an unpredictable angiogenic potential. Because MPCs show resistance to trypsin digestion, these cells are expected to be lost during subsequent passages reducing progressively the angiogenic potential of the sub-cultures. Not surprisingly, the most successful endothelial differentiation protocols have been obtained from early passages of MSC's cultures (Oswald et al., 2004; Fan et al., 2011; Janeczek Portalska et al., 2012). Conversely, protocols specific for endothelial differentiation may commit a pure population of MPCs into homogeneous clones of MSCs. The clinical utility of BM-derived cells will be improved by a more precise phenotypization able to distinguish MPCs from *early* and *late* MSCs.

Recently, Frenette's group demonstrated in the hematopoietic niche the presence of Nestin-positive bone marrow cells with mesengenic potential of differentiation. Using *Nes-Gfp* transgenic mice, authors identified a highly selected fraction of

Table 1 | *Bona fide* multipotent progenitor cells of bone marrow and adipose tissues.

Acronym	MSCs	MPCs	Pericytes	ARCs	APCs	TBLCs
Phenotype						
CD90	+	−	+	+	+	+
CD105	+	+	+	+	+	+
CD73	+	−				
Nestin	±	+		±		
CD31	−	+	−	−	−	−
CD146	±	−	+	−	−	±
CD271	+	−	±			+
CD34	−	−	−	+	+	−
Distribution	Bone marrow (Pittenger et al., 1999), adipose tissue (Zuk et al., 2001) and many others tissue cultures (da Silva Meirelles et al., 2006)	Bone marrow cultures (Petrini et al., 2009)	Sub-endothelium of vessels and microvessels in bone marrow (Tormin et al., 2011) and adipose tissue (Corselli et al., 2012)	Adventitia of vessels and microvessels in bone marrow (Sacchetti et al., 2007)	Adventitia of vessel and microvessel in adipose tissue (Tormin et al., 2011; Corselli et al., 2012)	Adjacent to trabecular bone (Tormin et al., 2011)
Role in angiogenesis	Controversial	Sprouting and direct differentiation into ECs	Stabilization of new formed vasculature	Mural cells and endothelium support	Mural cells and endothelium support	Not involved

MSCs able to form the HSC niche (Méndez-Ferrer et al., 2010). *In vivo*, both in human and mouse, these cells are positive for PDGFRα, CD51 and Nestin expression and negative for CD45 CD31 CD235a (Ter119- in mice). Although the two types of BM stem cell can form a single niche, only a small fraction of Nestin+ cells exhibits MSC activity when tested in mesensphere or CFU-F assays (Méndez-Ferrer et al., 2010). Also for Nestin-positive cells, the protocols of isolation and expansion dramatically affect the composition of cell populations in culture; thus harsh isolation protocols will be needed to avoid the development of heterogeneous populations and allow the characterization and functional definition of these interesting cells. Further the co-expression of PDGFRα and CD51 identifies a subset (about 60%) of Nestin-positive cells with an enriched potential to form HSC niches and to perform mesenchymal differentiation (Pinho et al., 2013). Limited data are available regarding the angiogenic potential of Nestin-positive cells. Because PDGFRα+ CD51+ hematopoietic-supporting stromal cells do not express CD45 (a hematopoietic marker) and CD31 (an endothelial marker), it can be hypothesized the presence of an additional population of Nestin-positive CD31-positive cells not described in the previous analysis.

CONCLUSIONS

Although MSCs have been largely studied for their interesting applications in clinical trials, these cells have not been fully characterized because of the lack of standardized protocols between different laboratories. Controversies remain, and several aspects of MSC biology are still unclear. The heterogeneity and morpho-functional variability of MSC cell preparations could explain most of the conflicting data in the literature. Together with the effects of culture conditions that can indeed select, or simply promote, particular subpopulations of MSC-like cells (**Table 1**), the described possible multiple origins of MSCs contribute to the confusing interpretation of the experimental data. More stringent phenotypization criteria may help to prevent this issue. Recently, perivascular localization of MSC precursors may explain their presence in a wide range of tissues and organs and suggests some angiogenic potential.

Therefore, we hypothesize that the presence of the recently described Mesodermal Progenitor Cells (MPCs) could be responsible for the controversial data regarding angiogenic potential of MSC cultures. Although these cells can be co-isolated with MSC culture, different protocols may determine a different yield of MPCs.

The discovery of Nestin-positive CD31-positive MPCs supports their role as MSC ancestors in human bone marrow and indicates the *tunica intima* as a possible source of MSCs.

Further studies are needed to deeply investigate the MPC biology and confirm their anatomical home in human bone marrow. Nonetheless, the identification of MPCs suggests the opportunity of a revision of the MSC definition in order to achieve their expected clinical utility (Keating, 2012).

Finally, MPCs represent a valuable cell population for the proof of new concepts in tissue engineering, where the neo-vascularization plays a crucial role in the establishment of successful therapies. Future studies evaluating MPC-based therapies will take advantage of their mesengenic and angiogenic potential in order to regenerate skeletal tissues and support their growth with a newly formed vasculature.

AUTHOR CONTRIBUTIONS

Simone Pacini: Literature review and article draft. Iacopo Petrini: revision.

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In vitro alteration of physiological parameters do not hamper the growth of human multipotent vascular wall-mesenchymal stem cells

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Background: Mesenchymal stem cells (MSCs) with multilineage potential and anti-inflammatory property can be isolated from different human tissues, representing promising candidates in regenerative medicine. Despite the common criteria of characterization, many factors contribute to MSC heterogeneity (i.e., tissue origin, coexistence of cell subsets at different stage of differentiation, epigenetic) and no standard methods have been approved to characterize MSCs in cell culture.

Aim: The present study aimed to test whether MSCs resist adverse chemical and physical culture conditions, surviving MSC subpopulations are endowed with the stemness abilities; to characterize MMP expression in AAA-MSCs under the adverse experimental conditions.

Methods and Results: MSCs enzymatically isolated from human abdominal aortic aneurysm (AAA-MSCs) were exposed to media acidification, hypoxia, starving, drying and hypothermia through the following strategies: (1) low-density seeding in closed flasks; (2) exposure to a chemical hypoxia inducer, cobalt chloride; (3) exposure to a dry environment with growing medium deprivation and culture at 4°C. None of these conditions affected MSC viability and stemness profile, as evidenced by NANOG, OCT-4, and SOX-2 mRNA expression in surviving cells. A significant MMP-9 decrease, especially when AAA-MSCs were exposed to hypothermia, was associated with stress resistant stem cells.

Conclusions: AAA-MSCs survive to extremely adverse culture conditions, keeping their morphology and stemness features. Besides MMP-9 role in pathological tissue remodeling, this protease may be related to MSC survival. Future studies on MSCs derived from other tissues will be necessary to refine our culture protocol, which can represent an empirical method to demonstrate MSC stemness, with potential implications for their clinical use.

Keywords: MSCs, survival, hypoxia, dry culture, hypothermia, MMP-9, anhydrobiosis

Introduction

The concept of "cell" originated in 1667 from Hooke's findings and the first attempt to cultivate cells from animal tissues was conducted in 1885 by Roux, who isolated the chick medullary plate and kept it alive for some days in saline solution. Two years later, Arnold cultivated the first leucocytes and many cell culture studies followed (White, 1954). First recorded *in-vitro* functional cell line was cultured in 1955 by Ted Puck and named HeLa (Puck et al., 1956). Since then, there is a large choice of commercial cell line selection in many repository: about 4000 cell lines are maintained by the American Type Culture Collection (ATCC). Currently, many laboratories routinely isolate and grow differentiated as well as stem cells from healthy and pathological tissues to establish primary cell cultures.

Our research group, in the last 15 years, succeeded in establishing viable cell lines with characteristics of mesenchymal stem cells (MSCs) from different vascular tissues. The vascular wall-MSC (VW-MCSs) are negative for CD45 and co-express CD44, CD90, and CD105 molecules, like the bone marrow-derived MSCs (Pasquinelli et al., 2007). Further, VW-MSCs express the neuronal stem cell intermediate filament, nestin, the stemness markers stromal precursor antigen-1 (Stro-1), sex-determining region Y-box-2 (SOX-2 or SRY), neurogenic locus notch homolog protein-1 (Notch-1) and octamer binding transcription factor-4 (Oct-4), while maintaining the ability to differentiate into mesengenic as well as vascular, i.e., endothelial and leiomyogenic, lineages, when cultured in appropriate induction media (Pasquinelli et al., 2010; Valente et al., 2014).

A review of the literature indicates that 130 years after stem cell discovery, the specificity of stem cell antigenic profile is still debated (Vasuri et al., 2014) and the identification of stem cells in culture is even more elusive due to the lack of a standard and coherent methodology to select them in culture (Hart, 2014).

Usually stem cells, that are believed to be highly glycolytic (Simsek et al., 2010), are expanded in closed systems (i.e., flasks) with glucose-bicarbonate buffered media as Eagle's MEM, at 37°C, 5% CO_2; they are seeded at a proper density unit that is chosen by taking into account the cell dimensions, the growth kinetics and the flask surface.

Conversely, some sporadic studies have proven that both glucose and CO_2 have detrimental effects on stem cell growth: glucose induces mesenchymal/endothelial stem cell senescence and apoptosis, while the glucose level reduction increases stem cell proliferation and colony forming activity (Kränkel et al., 2005; Saki et al., 2013). In 1985, Barngrover modified the L-15 medium, that was tailored by Leibowitz to promote the growth of the less glycolytic cells (Leibovitz, 1963), by substituting galactose with fructose; this allowed to maintain an optimal pH and lactate/pyruvate ratio (Barngrover et al., 1985). Similarly, cell culture is conventionally performed at 5% CO_2 and 21% oxygen (O_2 atmospheric concentration; 151.2 mm Hg): these gas concentrations are far away from those measured *in vivo*; according to Souza (2007), oxygen levels range from 1% (7.2 mm Hg) in the bone marrow tissue to 12% in lungs (86.4 mm Hg). Accordingly, CO_2 and O_2 diffusion in flask strongly influences cells growth; low O_2 tension (i.e., quasi-physiological) stimulates human embryonic cell growth and cellular differentiation as well as low density seeding (Balin et al., 1984). Also hypothermia (4°C) was shown to affect stem cell behavior *in vitro*; hypothermia was used to select satellite muscle stem cells vs. fibroblast, and, most importantly, skeletal muscle stem cells remained in a dormancy state in human cadavers stored at 4°C for 2 weeks giving origin to myotubes after that *ex vivo* cultures have been established (Latil et al., 2012; Marg et al., 2014). These spotted observations indicate that stem cells may survive to a range of stressful conditions including low temperature, starving and hypoxia; even more interesting is the evidence that here we have given about the possibility of the stem cells to survive in an anhydrobiotic dry environment, i.e., in flasks without culture medium or any nutrient supplement.

In this paper the experimental adverse culture conditions described above were empirically replicated in our laboratory using a MSC population isolated from aortic aneurysm (AAA-MSCs), a human cell model characterized by expression of mesenchymal and stemness markers as well as matrix metalloproteinase-9 (MMP-9) upregulation (Ciavarella et al., 2015).

This study was aimed (i) to test extreme culture conditions in a model of MSCs isolated from abdominal aortic aneurysm (AAA-MSCs) (ii) to test AAA-MSC ability to survive to hypoxia, starving, anhydrobiosis and hypothermia, (iii) to analyze AAA-MSC transcriptional profile regarding stemness factors and MMPs.

Materials and Methods

Isolation and Culture of Human MSCs

AAA-MSCs were isolated from the abdominal aorta of three male patients who underwent surgical repair for aneurysm, after obtaining Local Ethic Committee Approval (APP-13-01). Aortic tissues were provided by the Vascular Surgery Unit, S. Orsola—Malpighi University Hospital (Bologna, Italy). AAA-MSC isolation and characterization were performed as previously described (Valente et al., 2014; Ciavarella et al., 2015). Briefly, 2 cm^2 sections of aneurysmal wall were enzymatically digested with 0.3 mg/mL Liberase type II (Liberase TM Research Grade, Roche) in serum-free Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich) at 37°C o/n in a rotor apparatus. The tissue homogenate was filtered through cell strainers of different size (100-70-40 μm) and centrifuged at 1200 rpm. Cell viability was assessed by Trypan Blue exclusion. AAA-MSCs at passage 0 were cultured in DMEM enriched with 20% Fetal Bovine Serum (FBS; SIGMA Aldrich) at 37°C under an atmosphere of 5% CO_2 and expanded *in vitro*.

The transcriptional profile of stemness genes, and MMP-2, MMP-9, was performed to evaluate whether the extreme culture protocols we set up could affect AAA-MSC gene expression.

AAA-MSCs at passage 3 were tested for their ability to survive in stressful *in vitro* conditions, consisting in the lack of oxygen and nutrients, through different unconventional strategies. As explained in the experimental design (**Figure 1**),

these strategies included cell culture in closed flasks, using an essential glucose-free medium (MEM) and the complete lack of media either at 4°C or 37°C.

Low-density Culture of AAA-MSCs in Minimum Essential Medium

AAA-MSCs were seeded at low density (200 cells/cm^2) in no-vented T25 flasks with closed cap to keep the cells in a hypoxic environment.

Cells were grown in 10 ml of Minimum Essential Medium (MEM, Gibco), a specific medium under the intellectual property of Prof. W. Thilly (LIMB, Dept. of Biological Engineering, MIT, Cambridge MA) and manufactured on-demand by Gibco. This medium is free of D-glucose, antibiotics and sodium bicarbonate and contains D-fructose. For *in vitro* analysis we added L-glutamine 4 mM and 10% FBS. Culture media were regularly changed each 10 days. After 3 days from seeding, we measured the pH of surnatant, making a comparison with the cells grown in conventional DMEM. At the end of the experimental protocol AAA-MSCs were processed for RNA extraction.

Hypoxic Culture of AAA-MSCs Using $CoCl_2$

As an alternative strategy to induce oxygen deprivation, we exposed AAA-MSCs to a chemical inducer of hypoxia, Cobalt (II) Chloride hexahydrate ($CoCl_2$, Sigma). $CoCl_2$ is a mimetic agent used *in vitro* to induce cellular responses mediated by hypoxia (Piret et al., 2002; Wu and Yonda, 2011).

AAA-MSCs at passage 3 were seeded at a density of 10^4/well in a 96-well plate in traditional DMEM supplemented with 10% FBS, following the standard cell culture procedures and after 24 h $CoCl_2$ was added. The treatment was performed for 24–48–72 h at increasing concentrations (0–100 μM–500 μM). After any treatment, cells were processed for RNA extraction.

Cell viability after $CoCl_2$ treatment was estimated by Sulforhodamine B (SRB) assay. After treatment, the cells were washed twice with PBS and fixed in 50% aqueous trichloroacetic acid (TCA) for 1 h at 4°C, rinsed several times with water and incubated with 50 μl/well SRB solution (0.4% in 1% acetic acid) for 30 min. SRB solution in excess was washed off by 1% acetic acid. The cells were incubated in 10 mM Tris for 20 min and the absorbance of each well was measured in a microplate reader (Bio-Rad, Hercules, CA, USA) at 570 nm. The results were expressed as a percentage of treated on controls (untreated cells).

AAA-MSC Dry Culture (4°C–37°C)

The third strategy consisted in inducing an anhydrobiotic condition by removing the culture media completely at low temperature. For this purpose, AAA-MSCs were deprived of culture media and kept at 4°C. In parallel, AAA-MSCs from the same sample were treated as described above and maintained at 37°C for 3 days. To ensure the complete environment dehydration, the flasks were housed in a vertical position. After 7 days DMEM 10% FBS was added, flasks were incubated at 37°C and after 14 days treated with trypsin and cells seeded until confluence. At the end of the experimental protocol AAA-MSCs were processed for RNA extraction.

Total RNA Extraction and cDNA Synthesis

AAA-MSCs grown in the described culture conditions, were processed for RNA extraction using TRIreagent (TRIzol reagent, Invitrogen, Italy) according to the manufacturer's instructions. One μg of total RNA was reverse transcribed in a 20 μL volume of reaction using High Capacity Reverse Transcription Kit (Applied Biosystems). RNA integrity and concentration were measured using a ND-1000 spectrophotometer (NanoDrop, Fisher Thermo, Wilmington, DE, USA). Reverse transcriptase PCR was performed on all RNA samples with an absorbance

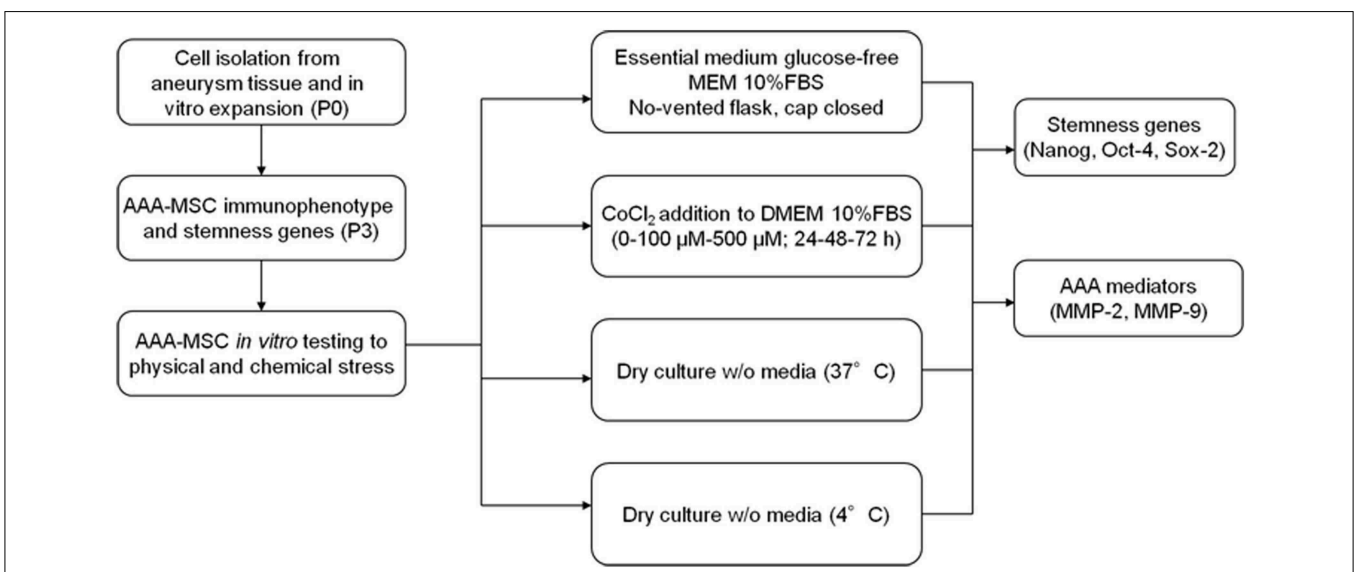

FIGURE 1 | Experimental design.

(260/280) ratio between 1,8 and 2,2. cDNA was synthesized from 2 μg of total RNA by using High Capacity Reverse Transcription Kit (Life Technologies).

RT–PCR Stemness Gene Expression Analysis

Stemness gene primers are listed in **Table 1**. The PCR primers were purchased from Invitrogen and SIGMA Aldrich. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene to value the cDNA quality. All PCR products were analyzed on 2% agarose gel electrophoresis with Tris-acetate-EDTA buffer 1X, stained with ethidium bromide incorporation and photographed under ultraviolet light. A 100 bp DNA ladder was loaded to allow PCR product size identification. The gel was subjected to electrophoresis at a constant 100 V for 45 min.

Quantitative Real-time Polymerase Chain Reaction

Real Time Polymerase Chain Reaction was performed to investigate the transcriptional levels of MMPs and apoptotic genes in AAA-MSCs exposed to the extreme culture conditions. Real Time PCR analysis was carried out in a Gene Amp 7000 Sequence Detection System (Applied Biosystems) using the TaqMan approach for the β-glucuronidase (GUS), B-cell lymphoma-2 (BCL-2), BCL-2 associated protein (BAX) genes (Applied Biosystems) and SYBR green approach for all other genes, using specific couples of primers, purchased from SIGMA-Aldrich: MMP-2 (FWD 5′-CCCCAAAACGGACAAA GAG-3′, REV 5′-CTTCAGCACAAACAGGTTGC-3, MMP-9 (FWD 5′-GAACCAATCTCACCGACCAG-3′, REV 5′-GCCA CCCGAGTGTAACCAT-3′).

Each assay was executed in triplicate and target gene expression was normalized to the housekeeping GUS gene. The final results were determined by the comparative 2^-DDCt method (Livak and Schmittgen, 2001) where DDCT = [CT Target − CT Gus] treated AAA-MSCs -[CT Target − CT Gus] control AAA-MSCs. Results were expressed as fold changes relative to AAA-MSCs grown in standard culture conditions as controls.

TABLE 1 | List of primers used for RT-PCR detection of stem cells transcriptional factors in AAA-MSCs exposed to stressful *in vitro* cultures.

Gene	Primer sequence	Product size (bp)	T (°C)
GAPDH	FWD 5′-ACCACAGTCCATGCCATCAC-3′ REV 5′-TCCACCACCCTGTTGCTGTA-3′	452	61
NANOG	FWD 5′-AAGGCCTCAGCACCTACCTA-3′ REV 5′-ACATTAAGGCCTTCCCCAGC-3′	326	58
OCT-4	FWD a 5′-CTCCTGGAGGGCCAGGAATC-3′	380	62
	FWD b 5′-ATGCATGAGTCAGTGAACAG-3′ REV 5′-CCACATCGGCCTGTGTATAT-3′	402	
SOX-2	FWD 5′ACCGGCGGCAACCAGAAGAACAG-3′ REV 5′-GCGCCGCGGCCGGTATTTAT-3′	208	62

bp, pair of bases; °C, centigrade degrees.

Immunofluorescence Assay

Immunofluorescence was performed on AAA-MSCs exposed to $CoCl_2$ to detect MMP-9 protein and its alteration following hypoxic induction. Briefly, 4×10^4 AAA-MSCs were cultured on collagen biocoated slide chambers (BD Bioscence, San Jose, CA, USA) and after 24 h $CoCl_2$ was added to cultures according to the concentration range used for all the other experiments: 0–100 μM–500 μM for 24, 48, and 72 h. at the end of the treatment, cells were gently were washed with PBS and fixed with cold absolute methanol, for 10 min air dry at room temperature. Fixed cells were then blocked in 1% bovine serum albumin (BSA) in PBS solution and donkey serum, specific for the secondary antibody species, for 30 min at room temperature.

After blocking, cells were incubated with primary antibody anti-MMP-9 (1:500, Cell Signaling) for 1 h at 37°C. Samples were then washed with PBS and incubated with Alexa Fluor 546 (1:250; Invitrogen, Carlsbad, CA, USA) secondary antibody in 1% bovine serum albumin in PBS for 1 h at 37°C in the dark. Finally, after washes, the samples were mounted and nuclei counterstained with Pro Long anti-fade reagent with DAPI (Molecular Probes, Milan, Italy). Images were acquired by a Leica DMI4000 B inverted fluorescence microscope (Leica Microsystems, Milan, Italy) at × 20 magnification.

Statistical Analysis

All experiments were performed in triplicate. Results were analyzed by GraphPad Prism 5 statistical software (GraphPad Software Inc) and are expressed as mean ± standard deviation. Statistical analysis was performed using *t*-test and Two-Way ANOVA test for comparison between more than two groups, followed by Bonferroni *post-hoc* test. Results were considered statistically significant at the 95% confidence level ($p < 0.05$).

Results

Properties of MSC Isolated from Aneurysm Wall (n = 4)

AAA-MSCs were obtained from 4 aortic tissues kindly provided by the Vascular Surgery Unit, S. Orsola—Malpighi University Hospital (Bologna, Italy). Clinical data of patients enrolled in this study are described in **Table 2**.

AAA-MSCs were adherent to plastic substrate, exhibiting a typical spindle-shaped morphology (**Figure 2A**), expressed mesenchymal markers on their surface and stemness genes Oct-4, Nanog, SOX-2. A 400-fold increased MMP-9 expression both at the mRNA and the protein level was observed in AAA-MSCs when compared to MSCs isolated from healthy aorta (Ciavarella et al., 2015).

AAA-MSCs Are Able to Survive Under Different Stress Culture Conditions

AAA-MSCs grown in hypoxic condition and in glucose-free medium showed a reduction of the supernatant pH at day 3, compared to AAA-MSCs derived from the same aortic sample and cultured according to the standard procedures (pH 6.4

in MEM vs. pH 7.5 in the control). The pH acidification did not influence the cell viability, indeed we noticed clusters of surviving AAA-MSCs at day 7 (**Figure 2B**) and after 2 weeks a monolayer of cells was detectable; no sign of cell death or suffering was simultaneously observed (**Figure 2C**). Thus, the AAA-MSCs demonstrated high resistance to acid environment and glucose deprivation.

Oxygen deprivation was also achieved through the use of $CoCl_2$ that was added to AAA-MSCs cultures in traditional DMEM with 10% FBS. Interestingly, after 24 h exposure to 100 μM $CoCl_2$ we did not observe any change in cell viability as measured through the Trypan blue exclusion assay. This result demonstrated a higher cell resistance to the $CoCl_2$ cytotoxic concentration, as documented in the literature (Wu and Yonda, 2011). Sulforhodamine B assay on AAA-MSCs exposed to higher $CoCl_2$ concentrations for a longer time, showed a dose- and time-dependent reduced cell viability (**Figure 4A**). A significant decrease of cell growth was observed when AAA-MSCs were exposed to 500 μM $CoCl_2$ for 48 h (the relative absorbance in $CoCl_2$-treated AAA-MSCs was 0.58, compared to 0.9 in untreated control; $p < 0.001$, Two-Way ANOVA test, followed by Bonferroni *post-hoc* test) and 72 h (the relative absorbance in $CoCl_2$-treated AAA-MSCs was 0.63, compared to 0.9 in untreated control; $p < 0.05$, Two-Way ANOVA test, followed by Bonferroni *post-hoc* test).

The third drastic strategy consisted in the complete lack of culture media (dry environment) and the maintenance at low temperature. **Figure 2** shows the morphological aspect of AAA-MSCs after culture media discard: cells encountered a volume reduction and lost their typical morphology (**Figures 2D,E**,

TABLE 2 | Clinical characteristics of abdominal aortic aneurysm patients.

Clinical data of AAA patients ($n = 4$)	
Age (mean ± S.D. and range)	68.75 ± 5.7 (61–74)
Sex	Males
DAAA (mean ± S.D. and range)	66.25 ± 13 mm (52–70)
Cholesterol (mean ± S.D. and range)	155 ± 28 mg/dl (116–178)
LDL (mean ± S.D. and range)	84 ± 27 mg/dl (49–110)
HDL (mean ± S.D. and range)	51.3 ± 7.8 mg/dl (43–60)
Triglycerides (mean ± S.D. and range)	109 ± 34.28 mg/dl (99–155)
Smoking (%)	75
Hypertension (%)	100
Diabetes (%)	0
Statins (%)	50

DAAA, abdominal aortic aneurysm diameter; LDL, low density lipoprotein; HDL, high density lipoprotein.

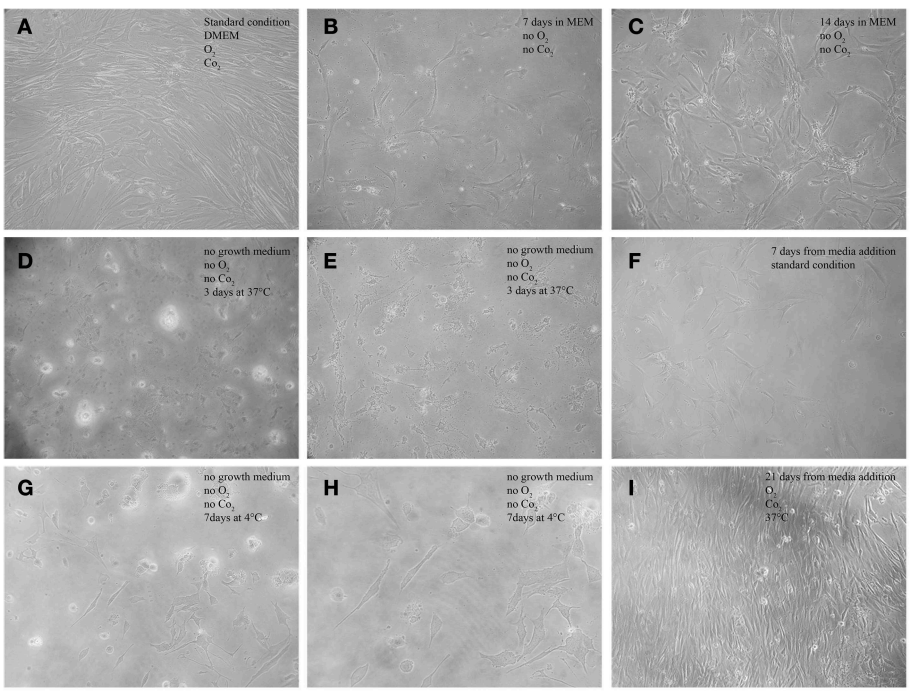

FIGURE 2 | Morphological features of AAA-MSCs exposed to hypoxia, nutrient deprivation, dry culture, and/or cold temperature. (A) Typical spindle-shape morphology of confluent AAA-MSCs at passage 3, grown in standard culture conditions; **(B)** Clusters of AAA-MSCs cultured in no-vented closed flasks using MEM glucose-free at 7 days and **(C)** at 14 days from seeding, where a cell monolayer is visible; **(D,E)** AAA-MSCs subjected to media deprivation lost their typical structure, cytoplasmic prolongation and plasma membrane; **(F)** 7 days from media addition, an amount of survival cells was detected; **(G,H)** AAA-MSCs after media deprivation and 7 days at 4°C showed a volume contraction and the typical cell shape was lost; **(I)** after traditional media addition, AAA-MSCs restored their features and reached confluence; (**A–G,I**: 100x magnification; H: 200x magnification).

37°C; **Figures 2G,H** 4°C). When traditional media was added, cells were cultured according to the standard procedures, and after two passages AAA-MSCs that have restored their original phenotype were observed (**Figures 2F,I**). Hypothermia was more effective than the 37°C alternative in selecting highly stress resistant AAA-MSCs: after 7 days at 4°C, cell viability was strongly decreased, as no cells were detected in the flask under the light microscope. After passaging the cells in culture, we could notice an expanded colony of 8 cm^2, suggesting that only few cells could survive and be expanded at low temperatures.

Transcriptional Profile of AAA-MSCs Exposed to Stress in *in-vitro* Cultures

As previously shown, AAA-MSCs express transcriptional factors involved in the survival and self-renewal programs typical of stem cells. Here we evaluated the expression of stemness genes NANOG, OCT-4 and SOX-2 after AAA-MSCs have been exposed to the culture conditions described above. No differences in the molecular expression of NANOG, OCT-4 and SOX-2 were observed at RT-PCR in AAA-MSCs exposed to low oxygen levels, media removal and low temperature (**Figure 3A**); the stemness profile of AAA-MSCs was not affected by the adverse culture conditions.

Transcriptional Analysis of MMP-2 and MMP-9 in AAA-MSCs Exposed to Stress in *in-vitro* Cultures

After the exposure to the extreme culture conditions, AAA-MSCs were processed to evaluate the mRNA production of the AAA molecular mediators, MMP-2 and MMP-9, the latter having been found 400-fold up-regulated in AAA-MSCs when compared to MSCs isolated from healthy control aorta. MMP-2 mRNA showed a weak increase in AAA-MSCs following hypoxia (0.8-fold increased, $p > 0.05$, paired t-test) and nutrient deprivation at 37°C (0.7-fold increased, $p > 0.05$, paired t-test), in comparison to AAA-MSCs grown according to the standard protocol, but no significant differences were observed; a significant increase was recorded after nutrient deprivation and culture at 4°C (0.7-fold higher compared to controls, $p < 0.05$, paired t-test) (**Figure 3B**). A significant decrease of MMP-9 was observed in AAA-MSCs cultured in a dry environment both at 37°C (5.5-fold decreased in comparison to control AAA-MSCs, $p = 0.03$, paired t-test) and 4°C cultures (13.6-fold decreased in comparison to control AAA-MSCs, $p = 0.02$, paired t-test) (**Figure 3C**).

Molecular Profile of AAA-MSCs Exposed to $CoCl_2$–Induced Hypoxia

RT-PCR revealed that stemness genes NANOG, OCT-4, and SOX-2 were expressed in AAA-MSCs grown in the hypoxic environment induced by cobalt chloride (**Figure 4B**), thus confirming that hypoxia did not influence their stemness genetic profile.

A significant decrease of MMP-9 transcription levels was observed when AAA-MSCs were exposed to $CoCl_2$ for 48 h and 72 h (0.65 after 48 h and 0.75 after 72 h-fold decreased, compared to the untreated AAA-MSCs) (**Figure 5A**). In addition, MMP-9 protein decrease was demonstrated by immunofluorescence detection on AAA-MSCs. Almost no signal was detected in cells treated with 500 μM $CoCl_2$ for 48 and 72 h (**Figure 5B**).

Moreover no significant changes in mRNA expression of BCL-2 and BAX were observed between MSC control and MSCs treated with $CoCl_2$, meaning no increase of death cell percentage (**Figures 5C,D**).

Discussion

In the present study we demonstrate that mesenchymal stem cells (MSCs) can grow in a non-conventional manner, survive when exposed to extreme adverse conditions, exhibiting high resistance to media acidification, glucose, oxygen, and nutrient deprivation,

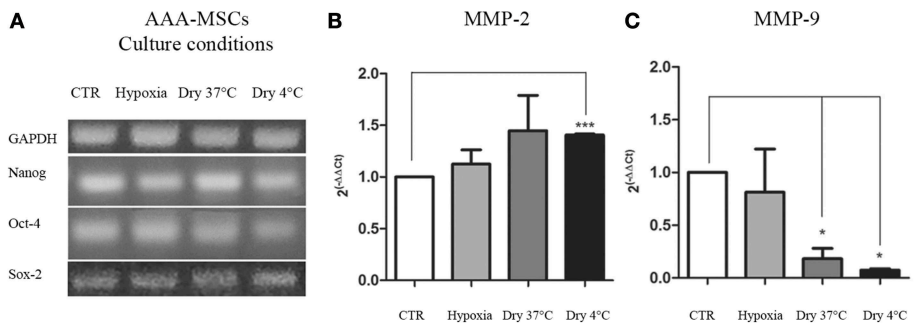

FIGURE 3 | Transcriptional profile of AAA-MSCs exposed to hypoxia, nutrient deprivation, dry culture and/or cold temperature. **(A)** NANOG, OCT-4 and SOX-2 expression was evaluated by RT-PCR and revealed that the oxygen deprivation and the growth medium removal (at 37 and 4°C) did not affect the transcription of genes involved in self-renewal and survival processes. GAPDH was used as housekeeping gene. Results are representative of three independent experiments. **(B)** MMP-2 and **(C)** MMP-9 mRNA expression in AAA-MSCs exposed to the described culture protocols. A significant increase of MMP-2 transcript expression was observed only in AAA-MSCs grown in absence of media at cold temperature; MMP-9 levels of expression decreased in all the experimental conditions, especially when cells were subjected to a dry environment at 4°C. β-glucuronidase gene was used as housekeeping. Results are expressed as fold changes relative to AAA-MSCs grown according to the standard culture protocol (DMEM 20% FBS, 37°C, vented flask). Values are represented as mean ± standard deviation and are representative of at least three independent experiments carried out in triplicate. (*$p < 0.05$; ***$p < 0.001$ paired t-test).

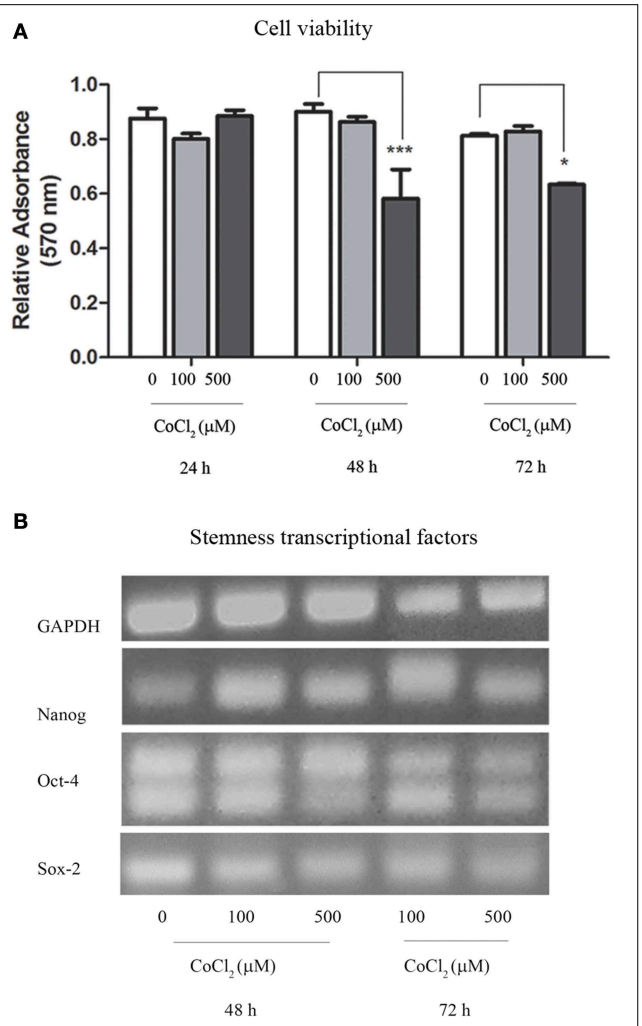

FIGURE 4 | Cytotoxic assay and transcriptional profile of AAA-MSCs exposed to $CoCl_2$. (A) AAA-MSC viability after 24–48–72 h exposure to $CoCl_2$ (0-100-500 μM), measured by sulforhodamine B assay. Values are represented as mean ± standard deviation and are representative of at least three independent experiments (*$p < 0.05$; ***$p < 0.001$; Two-Way ANOVA test, followed by Bonferroni post-hoc test). **(B)** mRNA expression of NANOG, OCT-4 and SOX-2 after 48–72 h exposure to $CoCl_2$ (0-100-500 μM), performed by RT-PCR. GAPDH was used as housekeeping gene. Results are representative of three independent experiments.

dry culture, and hypothermia while keeping their morphology and stemness features.

MSCs obtained from human tissues are of great interest for regenerative medicine, thanks to their self-renewal property, multilineage potential (Conget and Minguell, 1999; Pittenger et al., 1999), and immunoregolatory abilities (Ryan et al., 2005).

In 2006, Dominici et al., defined the minimal criteria for MSC characterization: adherence to plastic, specific surface antigen expression, differentiation into adipocytes, osteoblasts and chondroblasts following specific *in vitro* stimulation (Dominici et al., 2006). MSCs can be isolated from various tissues, including adipose tissue (Zuk et al., 2002), umbilical cord blood (Erices et al., 2000), skeletal muscle (Asakura et al., 2001), synovial membrane (De Bari et al., 2001), arterial wall (Pasquinelli et al., 2007). This factor implies that MSCs of different tissue origins show high variability; in addition, many subsets of cells at distinct stage of their differentiation and commitment coexist in the same MSC pool (Karystinou et al., 2009), increasing the MSC heterogeneity and making the MSC application for clinical use difficult.

We investigated a tissue culture model of MSCs isolated from human abdominal aorta with aneurysm disease and assayed three unconventional approaches to culture them, consisting of hypoxia, starving and cold temperature. Developing these conditions generally require specific facilities and, in our laboratory, it has been executed through empirical approaches to easily reproduce them. These methods are supposed to select the stemness compartment of the AAA-MSC population, targeting the surviving cell population that have the highest resistance to stressful conditions, typical features of stem cells at an undifferentiated status.

In the first condition, AAA-MSCs were cultured at low density in an essential glucose-free growth medium, in closed flasks to ensure the O_2 deprivation. We interestingly noticed that these stressful expedients did not drastically reduce the cell number, despite the supernatant acidification; on the contrary, after 21 days from seeding, AAA-MSCs reached confluence. In addition, we used $CoCl_2$, as a chemical inducer of hypoxia, widely applied in hypoxic culture systems. We interestingly observed that AAA-MSCs could resist exposure to $CoCl_2$at concentrations that normally result cytotoxic, as shown by the viability assay. A significant cell viability decrease derived only at longer time of exposure (48 and 72 h). These data are consistent with literature indeed hypoxia has been shown to contribute to the undifferentiated status of human MSCs (Basciano et al., 2011).

As hypoxia, both physically and chemically induced, did not influence the AAA-MSC growth, we further investigated the effect of a dry culture in which an anhydrobiotic condition was simulated by the complete removing of the culture media from the flasks that were housed in vertical position at low temperature; the dry culture induces a suffering condition due to the lack of the ordinary nutrient supply, necessary to the cell growing. At the end of the process, we observed the formation of a cell cluster, with a colony aspect, suggesting that clones with high resistance to the extreme environment was selected from the whole cell population. Hypoxia, dry culture as well as hypothermia, did not affect the stemness genes NANOG (Chambers et al., 2003; Hart et al., 2004), OCT-4 and SOX-2 (Richards et al., 2004) expression in our cell model, thus demonstrating that surviving cells express well-established markers of stemness and pluripotency. Surprisingly, MMP-9 was almost undetectable at the mRNA level and a reduced signal intensity was noticed on AAA-MSCx esposed to $CoCl_2$ by immunofluorescence assay; this reduced expression was not related to a proportional decrease in cell viability. As recently reported by Ciavarella et al., AAA-MSCs usually present a significant increase of MMP-9 transcript and protein when compared to healthy aortic MSCs (Ciavarella et al., 2015); this increased protein expression was related to the MMP role as molecular mediators of aneurysm pathogenesis. The

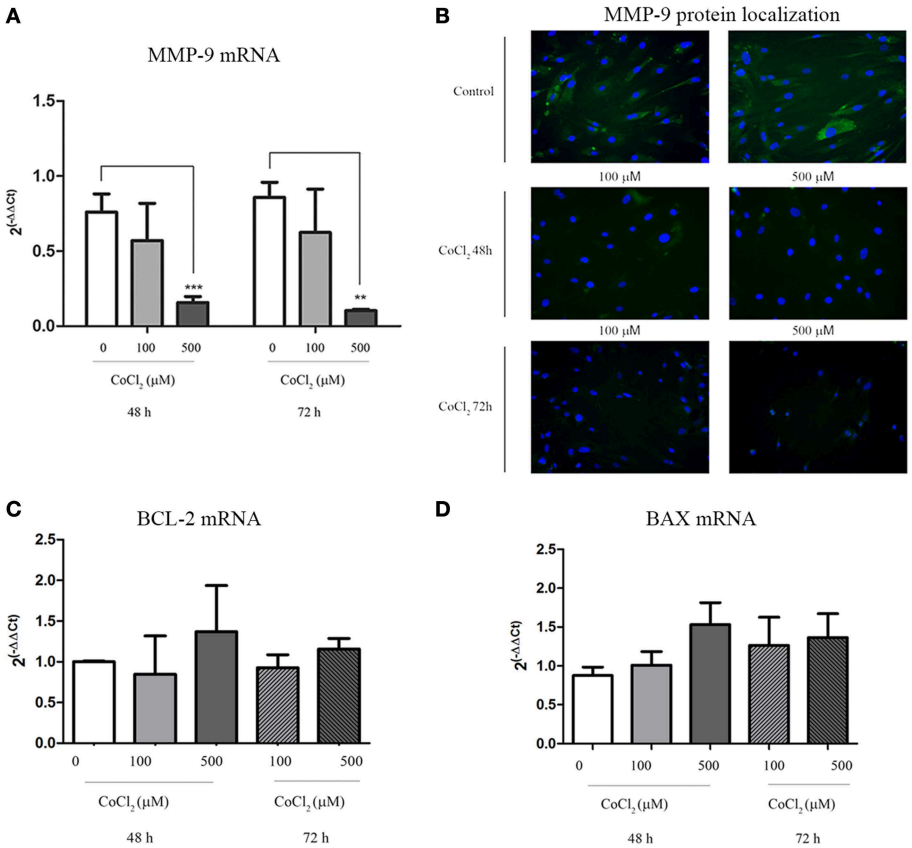

FIGURE 5 | MMP-9 and BCL-2/BAX expression in AAA-MSCs after $CoCl_2$ treatment. (A) MMP-9 mRNA analysis on AAA-MSCs after 48–72 h exposure to $CoCl_2$ (0-100-500 μM) evaluated by Real-Time PCR. **(B)** Immunofluorescence micrographs representing MMP-9 (green) expression treated with $COCl_2$. In untreated AAA-MSCs the MMP-9 protein was revealed in the cell cytoplasm. Following exposure to $COCl_2$ the MMP-9 protein was rarely detectable in cell cytoplasm, especially after 72 h treatment. Nuclei (blue) were counterstained with DAPI. **(C)** BCL-2 and **(D)** BAX mRNA analysis on AAA-MSCs after 48–72 h exposure to $CoCl_2$ (0–100-500 μM) evaluated by Real-Time PCR. β-glucuronidase gene was used as housekeeping. Results are expressed as fold changes relative to untreated AAA-MSCs. Values are represented as mean ± standard deviation and are representative of at least three independent experiments carried out in triplicate. (**$p < 0.01$; ***$p < 0.001$ paired *t*-test).

MMP-9 down regulation in AAA-MSCs exposed to the described protocols, suggest additional functions related to MSC survival and differentiation programs.

Many studies have evaluated the extracellular matrix (ECM) contribution in driving the stem cell fate; MMPs are a family of Ca^{2+} and Zn^{2+} endopeptidases that specifically cleave ECM components (Mannello et al., 2006) and are involved in several pathological processes characterized by matrix remodeling, as well as in cell and tissue development (Vu and Werb, 2000; Nagase et al., 2006; Mannello et al., 2006). MMPs and TIMPs are widely expressed by human MSCs (Silva et al., 2003; Panepucci et al., 2004; Ries et al., 2007), suggesting a crucial role also in self-renewal and/or differentiation (Mannello et al., 2006), although the mechanism is not completely elucidated. Heissig et al. (2002) demonstrated that MMP-9 is tightly associated with hematopoietic stem cell recruitment and mobilization from a quiescent niche to a proliferative microenvironment that allows differentiation, through the release of the soluble kit ligand.

Thus, MMP-9 may be not only involved in vascular and neoplastic disease progression through the ECM degradation, but it can also contribute to define MSC fate, regulating the differentiation and survival program. Future studies exploring the mechanisms involved in MMP-9 modulation will be necessary to elucidate its contribution to stem cell biology.

Conclusions

We have developed empirical strategies to select a pool of stress surviving MSCs, testing their resistance to extreme conditions, consisting of media acidification, glucose, oxygen, nutrient deprivation, dry culturing and hypothermia. We found that a fraction of MSCs is able to survive, without loosing stemness molecular characteristics. Interestingly, MMP-9 underwent a significant decrease of transcript levels. The hereby proposed protocol confirms that MSCs can grow in physiological-like oxygen concentration conditions, avoiding the use of the abnormally high atmospheric O_2 concentration and of CO_2, toxic for human tissues. A further advantage of the proposed culture method is that closed flask limits contact of cells with the external ambient thus preventing bacteria contamination, a frequent

event occurring when primary cultures are established. The possibility to grow successfully stem cells in an anhydrobiotic dry environment opens new questions about what are the surviving limits of undifferentiated mammalian cells. Most importantly, this methods do not require any specific or expensive facilities, so it can be performed in any laboratory with basic equipment and it may represent, once further refined, an effective alternative for MSC *in vitro* culture.

Limits of the Study and Perspectives

The proposed technique reflects the survival property of a MSC population isolated from a pathological source. Meanwhile, our study presents some limitations.

First of all, these experimental protocols need to be applied to an increased number of samples and tested in cell models representative of different tissue sources, other than vascular wall. The evaluation of stem cell differentiation potential under our conditions constitutes another topic to be investigated.

The MMP-9, beyond its participation to ECM remodeling that occurs during pathological processes, may be correlated to physiologic functions that characterize cell biology, like differentiation, survival and development. We have demonstrated that in a cell model that typically express high levels of MMP-9, the drastic alteration of pH, temperature and nutrient supply causes a significant reduction of its mRNA levels, not ascribed to cell death or suffering. This is a preliminary result that needs to be explored, evaluating what signaling mechanisms are involved.

Author Contributions

CC and SF conceived and designed the experiments, performed the experiments, analyzed the data and wrote the paper. SP designed the experiments, performed the experiments and analyzed the data. FV interpreted data and revised the paper. EG, AS, and AF provided samples, analyzed data and revised paper. EG and GP conceived and designed the experiments, analyzed the data, wrote the paper and revised the paper critically and gave final approval of the version to be published. All authors read and approved the final manuscript.

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Nanotopography Induced Human Bone Marrow *Mesangiogenic Progenitor Cells* (MPCs) to *Mesenchymal Stromal Cells* (MSCs) Transition

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Mesangiogenic progenitor cells (MPCs) are a very peculiar population of cells present in the human adult bone marrow, only recently discovered and characterized. Owing to their differentiation potential, MPCs can be considered progenitors for mesenchymal stromal cells (MSCs), and for this reason they potentially represent a promising cell population to apply for skeletal tissue regeneration applications. Here, we evaluate the effects of surface nanotopography on MPCs, considering the possibility that this specific physical stimulus alone can trigger MPC differentiation toward the mesenchymal lineage. In particular, we exploit nanogratings to deliver a mechanical, directional stimulus by contact interaction to promote cell morphological polarization and stretching. Following this interaction, we study the MPC-MSC transition by i. analyzing the change in cell morphotype by immunostaining of the key cell-adhesion structures and confocal fluorescence microscopy, and ii. quantifying the expression of cell-phenotype characterizing markers by flow cytometry. We demonstrate that the MPC mesengenic differentiation can be induced by the solely interaction with the NGs, in absence of any other external, chemical stimulus. This aspect is of particular interest in the case of multipotent progenitors as MPCs that, retaining both mesengenic and angiogenic potential, possess a high clinical appeal.

Keywords: mesangiogenic progenitor cells, mesenchymal stromal cells, bone marrow culture, polyethylene terephthalate, nanograting

INTRODUCTION

Mesenchymal stromal cells (MSCs) have been the object of extensive research for decades, due to their intrinsic clinical value. *Mesangiogenic progenitor cells* (MPCs), instead, were only recently discovered and characterized. They were firstly co-isolated, in different percentages, with MSCs in human adult bone marrow cultures applying autologous serum (Petrini et al., 2009); later,

the establishment of specific culture conditions selective for MPCs allowed the isolation of these cells with a high grade of purity (>95%). Specifically, when human bone marrow mononuclear cells (hBM-MNCs) are cultured in basal medium supplemented with human serum (HS) on hydrophobic surfaces, it is possible to select slow-cycling MPCs after 6 days of culture (Trombi et al., 2009) because this was the only cell population present in the initial preparation capable of attaching on hydrophobic plastic dishes.

Phenotypically, MPCs are easily distinguishable from MSCs for their fried egg-shape morphology and peculiar immuophenotype characterized by the positivity to CD18, CD31 and nestin as well as negativity to the MSC markers as CD73 and CD90. Interestingly, as MPCs initially showed to retain mesengenic, cardiomyogenic, and angiogenic potential, these cells were firstly named "*mesodermal*" progenitor cells. Later on, the mesengenic potential was definitively demonstrated and finely described in two steps of differentiation, once cultured in specific pre-formulated media for MSC expansion. Namely, MPCs firstly differentiate into slow cycling $CD90^{+}$/$nestin^{+}$ cells (*early*MSCs), activating non-canonical Wnt signaling, that generate exponentially growing MSC-like cells (*late*MSCs) after prolonged culture time (Fazzi et al., 2011). Similarly, angiogenic potential of MPCs was demonstrated. Also in this case the process involves two steps: first, MPCs are induced to VEGF-stimulated angiogenic sprouting; then they complete differentiation by forming capillary tube-like structures in 3D-cultures. Conversely, the cardiomyogenic potential was not definitively demonstrated, suggesting changing the MPC nomenclature into "*mesangiogenic* progenitor cells" (Montali et al., 2016) in spite of "*mesodermal*" progenitor cells. MPCs have been demonstrated deriving from a unique bone marrow cell population named *Pop#8* (Pacini et al., 2016). This cell population has been sorted from adult human bone marrow as $CD45^{low}CD31^{bright}CD64^{bright}CD14^{neg}$ and showed similarities to monocytoid progenitors. Moreover, MPC morphology and phenotype partially resemble macrophages suggesting that these mesangiogenic progenitors and their *in vivo* counterparts (*Pop#8*) could belong to the hemopoietic compartment. Similarly, plasticity of peripheral monocytes and macrophages has been demonstrated. Some groups reported evidences showing that these cells could differentiate into fibroblast-like and collagen-producing cells called "fibrocytes" which participate in tissue repair, mainly sustaining fibrosis (reviewed in Bucala, 2015). Nonetheless, demonstration of genuine mesengenic potential (osteogenic, adipogenic, and chondrogenic) of fibrocytes is still lacking, restricting their tissue repairing potential to scar formation, while sustaining to angiogenesis has been reported for these cells but only as paracrine secretion of pro-angiogenic factors (Grieb et al., 2011). Moreover, fibrocytes derived from peripheral $CD45^{bright}CD14^{+}$ monocytes also characterized for the expression of CD11b, C11c, CD13, and CD16 (Pilling et al., 2009), which have not been detected on bone marrow-derived $CD45^{low}CD14^{neg}$ *Pop#8*.

As MPCs can be considered progenitors for MSCs, they potentially represent a promising cell population to apply for skeletal tissue regeneration applications. Cell based medicinal products obtained by hBM-MNCs cultured in autologous serum were applied in the treatment of upper limb nonunions (Giannotti et al., 2013). In this paper, authors reported consistent percentages of MPCs (1–10%) within the expected MSC population, although not all of the conditions for MPC selection were complained. Nonetheless, it was suggested that the MPC fraction possibly contributed to the long-term healing reported in the treated patients. This hypothesis arose from the reported MPC plasticity suggesting that, once implanted, these cells could support osteogenesis differentiating into *early*MSCs, as well as contributing to the neo-vascularization of the engineered construct thank to their angiogenic potential.

In order to better investigate and exploit this possible therapeutic scenario in the field of orthopedics, further experiments should be performed *in vitro* in order to predict the MPC differentiation fate resulting from different physico-chemical stimulations that selectively mimic specific aspects of the *in vivo* micro-environments.

Bone tissue homeostasis represents a complex biological process finely regulated by humoral stimuli as hormones, growth factors and cytokines as well as by cell-cell and cell-matrix contact interactions (Florencio-Silva et al., 2015). Thus, optimal bone regenerative therapy should enhance mineralized tissue healing through enrichment of the bone defect with a micro/nanostructured matrix scaffold to support the wound, with cells that will give raise to osteoprogenitors and proper biochemical stimuli. Recently, a factor controlling the fate of many osteocompentent cells has been introduced, taking in consideration that micro- e nano-topography of the bone architectures could have a role in the regulation of the activity of the bone cells trough the activation of cellular mechanotransduction mechanisms mediated by adhesion molecules (Green et al., 1995; Zohar, 2012). In particular, this complex bone architecture resulted mainly sustained by collagen and hydroxyapatite (HA), which together form a highly aligned composite matrix that contribute to the toughness and strength of bone itself (Weiner et al., 1999; Kerschnitzki et al., 2011). Collagen triple helices are typically around 300 nm long and 1.5 nm in diameter (Weiner et al., 1999) conferring a linear topography to the bone structure at the nanoscale. Many studies reported the influence of nanotopography to the biology of osteoprogenitors (Dalby et al., 2007; McMurray et al., 2011; Janson et al., 2014) suggesting to control their differentiation applying nanostructured surfaces of orthopedic implants.

MPCs showed particular adhesion properties sustained by podosome-like structures, that were applied for the definition of a MPC selective culture method (Trombi et al., 2009). Further studies demonstrated that gelsolin-served F-actin podosomial structures were re-organizedn in paxillin-served F-actin stress fibers, during the mesengenic differentiation of MPCs (Pacini et al., 2013), suggesting that topographical stimuli could play a crucial role in the MPC fate.

The aim of this study is evaluating the effects of surface nanostructuring on MPCs, considering the possibility that the nanotopography alone can trigger the MPC differentiation toward the mesenchymal lineage. In particular, we exploited nanogratings to deliver a mechanical, directional stimulus by

contact interaction to promote cell morphological polarization and stretching. Following this interaction, we studied the MPC-MSC transition by (i) analyzing the change in cell morphotype by immunostaining of the key cell-adhesion structures and confocal fluorescence microscopy, and (ii) quantifying the expression of cell-phenotype characterizing markers by flow cytometry.

MATERIALS AND METHODS

Nanograting Fabrication

NGs were fabricated by thermal nanoimprinting lithography (NIL) on copolymer 2-norbornene ethylene cyclic olefin copolymer (COC) foils (IBIDI, Martinsried, Germany). COC was chosen because of its well-documented biocompatibility and optimal optical properties for high-resolution fluorescence microscopy. NIL is based on the combination of pressure and heat, which aids the transfer of the chosen pattern from a rigid mold to thermoplastic materials. Molds were fabricated by electron beam lithography (EBL) and reactive ion etching (RIE) techniques as previously reported (Cecchini et al., 2007). COC foils were imprinted using an Obducat Nanoimprint 24 system (Obducat, Sweden). After cleaning with nitrogen flow, the substrates were placed on top of the silicon molds and softened by raising the temperature up to 150°C. A pressure of 50 bar was then applied for 5 min before cooling down to 70°C, that is below the glass transition temperature of the copolymer (Tg = 134°C). Finally, the pressure was released and the mold detached from the substrate with a scalpel. The imprinted substrates were quality checked by optical microscopy and attached to the bottom of hollowed 35 mm Petri dishes by using silicone glue (RS Components RS692–524).

Donors and Sample Collection

Human bone marrow blood samples were collected, after written consent, from 12 patients (5 Male/6 Female, median age 64) during orthopedic surgery for hip replacement. The study has been performed according to the declaration of Helsinki and to the approval of the local ethical committee of "*Azienda Ospedaliero-Universitaria Pisana*." A 20 ml syringe containing 500 U.I. of heparin was used to aspirate 10 ml of fresh tissue immediately after femoral neck osteotomy and before femoral reaming. Samples were promptly sent to the cell culture facility and processed soon after.

MPC Preparation and Characterization

Cell Preparation

MPCs were obtained from hBM applying selective culture conditions, according to the previously reported method (Trombi et al., 2009; Montali et al., 2016). Briefly, bone marrow blood samples were diluted 1:4, carefully stratified on Ficoll-Paque™ Premium (GE Healthcare, Uppsala, Sweden) and centrifuge at 400 g for 30′. hBM mononuclear cells (hBM-MNCs) were collected at the interface of the density gradient, washed in Dulbecco's modified phosphate saline buffer (D-PBS, Thermo Scientific, Carlsbad, USA-CA) and resuspended in Dulbecco's modified Eagle medium (DMEM, Thermo Scientific) supplemented with 10% of pooled human type AB serum (PhABS, Lonza, Basel, Switzerland), 1:100 Glutamax™ (Thermo Scientific) and 100 μU/ml of penicillin and streptomycin (Thermo Scientific). Cell concentration and vitality were determined by Bürker hemocytometer. From 30 × 10^6 to 60 × 10^6 hBM-MNCs were then plated in a T75 culture flask for suspension cultures (Greiner Bio-one, Monroe, USA-NC) and incubated at 37°C and 5% CO_2 for 48 h in DMEM/10%PhABS. Cells in suspension were removed with the medium change and then adherent cell were maintained in culture for 6 days. At the end, medium was discard, flasks were washed with D-PBS and 1,5 ml of TrypLE® Select detaching solution (Thermo Scientific) was applied for 10′ to collect MPCs.

Flow Cytometry

3 × 10^5 freshly isolated MPCs were processed for immunophenotyping and incubated with anti-CD11c VioBlue®-conjugated, anti-CD18 PE-conjugated, anti-CD31 PE/Cy7-conjugated and anti-CD90 APC-conjugated (all from Miltenyi Biotec, Bergisch Gladbach, Germany) for 30′ at 4°C in the dark. Cells were then washed with MACSQuant® Running Buffer (Miltenyi Biotec) and resuspended in 500 μl of the same buffer for data acquisition in MACSQuant® Flow Cytometer (Miltenyi Biotec). Data were acquired and analyzed by MACSQuantify® analysis software (Miltenyi Biotec). Samples with percentage of $CD11c^+CD18^+CD31^+CD90^{neg}$ lower than 95% were excluded from the study.

Nestin Detection and F-Actin Organization

From 8 × 10^5 to 1.6 × 10^6 hBM-MNCs were seeded in 2-well culture chamber slides (Thermo Scientific) and cultured in DMEM/10%PhABS for 6 days, as described above. Cultures were then washed twice in D-PBS and MPCs were fixed in paraformaldehyde 4% for 15′. Permeabilization was performed by incubation in Triton X-100 0.5% (Sigma Aldrich) for 15′, after the removal of the fixative by extensive wash in D-PBS. Slides were blocked applying Image-iT™ FX signal enhancer (Thermo Scientific) and incubated with anti- human nestin monoclonal antibody (Abcam, Cambridge, UK) overnight. Antibody excess was removed by washing in Triton X-100 0.5% and slides were then incubated with AlexaFluor® 488-conjugated secondary antibody for 1 h. F-actin staining was performed by AlexaFluor® 555-conjugated phalloidin (Thermo Scientific) for 20′. The slides were finally mounted with ProLong® anti-fade reagent with DAPI and pictures were taken using an inverted fluorescence DM IRB microscope (Leica, Wetzlar, Germany) equipped with LAS AF image analysis software (Leica).

Mesengenic Differentiation by Pre-formulated Medium

Freshly detached MPCs were seeded in T75 flasks (20,000 cells/cm^2) and let adhere overnight in DMEM/10%PhABS. The day after the medium was changed with MesenPRO® RS medium (Thermo Scientific) and the cells were cultured until confluence (P1-MSCs). Cells were then detached by enzymatic digestion with TrypLE® Select and sub-cultured at 5000 cells/cm in new T75 flasks for flow cytometry analysis, and in 2-well

culture chamber slides for the detection of nestin (performed at confluence, P2-MSCs).

MPC Culture on T2 Nanogratings

Freshly isolated MPCs were resuspended and adjusted to 40,000 cells/ml in DMEM/10%PhABS, then a cell suspension volume of 500 μl was seeded into four T2 nanostructured inserts, in a drop. Cells were let adhere for 4 h, then additional fresh medium (1.5 ml) was added and cells were cultured for 7 days. In parallel, cultures have been performed on four "FLAT" inserts; additional cultures were also prepared in standard 6-wells plates for suspension cultures, in order to evaluate the spontaneous cell differentiation (marked as "CTRL"). For flow cytometry quantification of the mesengenic differentiation, the cells cultured onto the three different substrates were detached by TrypLE® Select digestion solution, washed in *MACS*Quant® Running Buffer (MiltenyiBiotec), and incubated with the monoclonal antibodies, as described above. The acquisition and analysis were performed applying dot-plot template set up for MPC/P2-MSCs analysis. Mesenchymal differentiation was evaluated by calculating the ratio between events with MSC-associated phenotype, detected in *R2* gate, and MPC-related events detected in *R1* gate (MSC/MPC).

Statistical Analysis

Data were reported as average value ± the standard error of the mean (mean ± SEM), obtained from at least three independent experiments. Data were statistically analyzed by GraphPad PRISM 6.00 program (GraphPad Software, San Diego, CA, USA). For parametric data, Student's *t*-test (unpaired, two-tailed) or One-Way ANOVA (Tukey's or Dunnett's multiple comparison test) analysis were used; the mean values obtained in each repeated experiment were assumed to be normally distributed about the true mean. Statistical significance refers to results where $p < 0.05$ was obtained. Further details on data representation and statistics are reported in the figure legends.

RESULTS

Nanograting Fabrication and Characterization

Three different substrates were chosen to study MPC differentiation toward the MSC phenotype. The substrates were produced starting form 200-μm-thick COC films by hot embossing (**Figure 1**), as detailed in the Materials and Methods section. This process provided us with nanostructured surfaces covering macroscopic areas (= 1 cm^2), well suitable for fluorescence microscopy and cell/molecular biology studies. Two surface geometries were fabricated, the first having an embossed nanograting (named T2) while the second was obtained with the same thermal and pressure cycle but using a flat mold (i.e., a silicon polished wafer). This last substrate is named FLAT and has been used as control condition for the T2 surface in each experiment. T2 is characterized by ridge and groove width of 1000 nm, and depth of 350 nm. All the COC surfaces were proven to be adhesive for MPCs without requiring any chemical functionalization but plasma activation. In this study and previously (Tonazzini et al., 2013; Jacchetti et al., 2014) we did not find any cytotoxicity originating by either the surface topographical modification or by the material itself. Finally, standard tissue culture Petri dishes were also used as control condition for the FLAT substrates in order to verify that the material itself did not cause the MPC-MSC transition. Summarizing, three different substrates were exploited for this study, the T2 and FLAT (both in COC), and a standard plastic Petri dish (named CTRL).

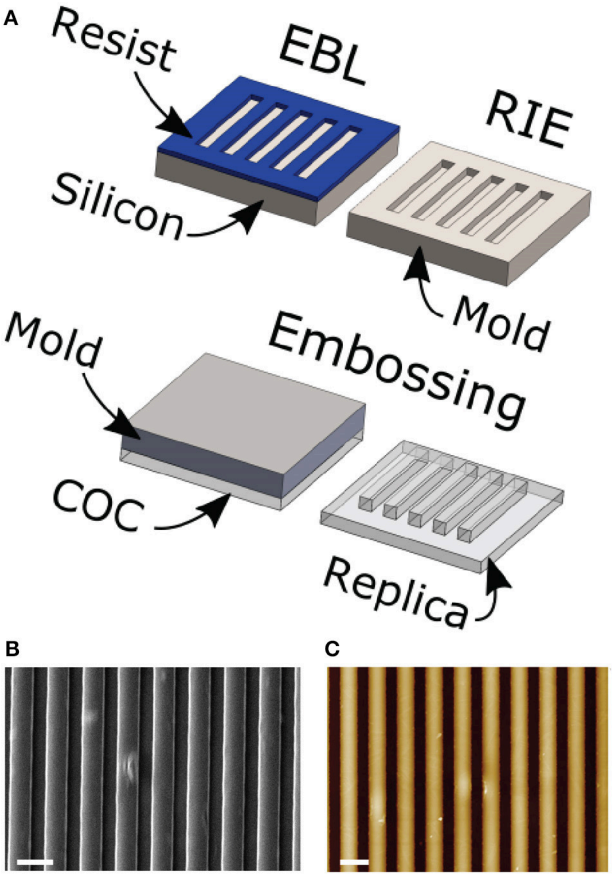

FIGURE 1 | (A) Scheme of the substrate fabrication process. COC foils are placed on nano/microstructured molds and imprinted following a single heating and pressure cycle. Scanning electron microscope **(B)** and atomic force microscope **(C)** representative images of a T2 substrate. Scale bars = 2 um; The color-scale in **(C)** goes from 0 (black) to 400 nm (white).

MPC Culture Characterization

All of the bone marrow samples could generate MPCs (**Figure 2A**), that were isolated by applying the previously reported method (Trombi et al., 2009; Montali et al., 2016). As expected, culturing hBM-MNCs for 6 days under selective culture conditions led to monomorphic cultures of adherent cells with a peculiar fried egg-shape morphology (**Figure 2A.1**). These cells were then characterized as MPCs for their intense expression of nestin (green in **Figure 2A.2**) and the typical dotted distribution of F-actin, that reveals numerous podosome-like structures (red in **Figure 2A.2**). Also flow cytometry confirmed the expected immunophenotype characterized by the positivity to CD31 (PECAM), CD18 (Integrin β2), CD11c (Integrin αX)

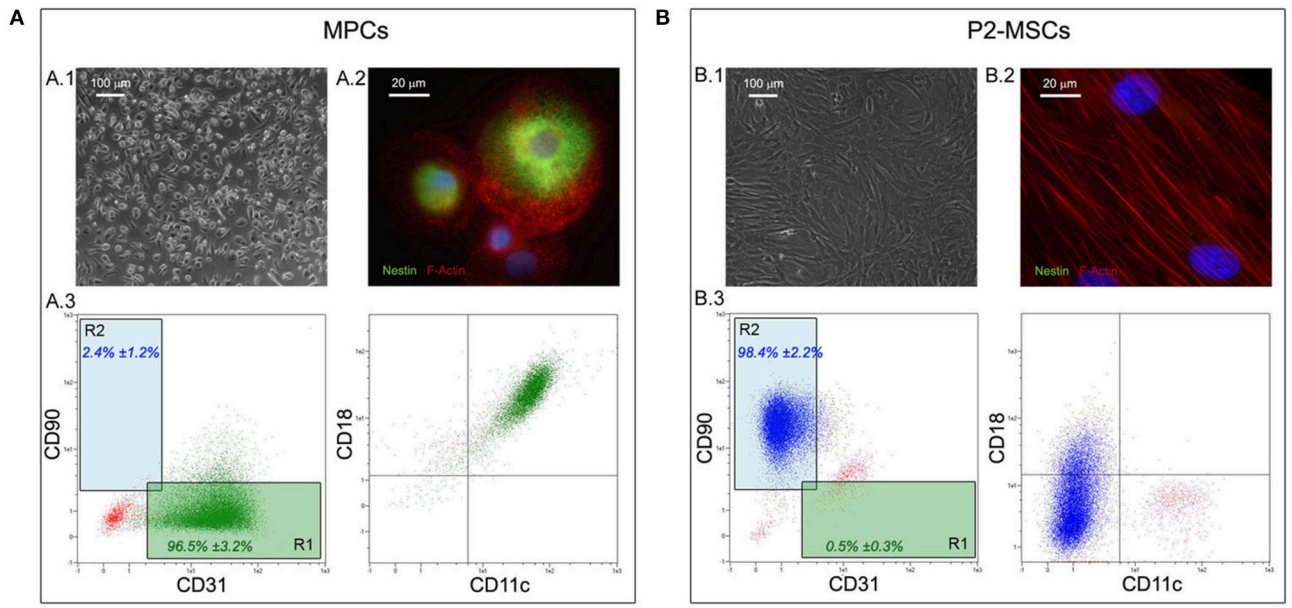

FIGURE 2 | Isolation and characterization of hBM-derived MPCs. Culturing hBM-MNCs for 6 days under MPC selective conditions lead to almost homogenous population of rounded and highly rifrangent adherent cells **(A.1)**. These cells were definitively characterized as MPCs for their intense positivity to nestin (green in **A.2**) and for the presence of numerous podosome-like structures characterized by the dotted distribution of F-Actin (red in **A.2**). Flow cytometry confirmed the MPC phenotype on approximately all analyzed cells **(A.3)**, which were displayed in the lower right quadrant of the CD90 vs. CD31 dot-plot ("R1" green box). After two passages under mesengenic differentiating conditions (P2-MSCs) the cultures were constituted by proliferating and fibroblastoid MSC-like cells **(B.1)** and nestin was no longer detected **(B.2)**. All MPC-related markers resulted undetectable by flow cytometry on P2-MSCs **(B.3)**, which expressed high levels of CD90, occupying the upper left quadrant of the CD90 vs. CD31 dot-plot ("R2" blue box).

and the lack of CD90 expression (**Figure 2A.3**). Flow cytometry was then applied to determine the purity of MPC cultures before subculture the cells on NGs. Most of the analyzed cell preparations revealed MPC percentages higher than 95% (mean 96.5 ± 3.2%, $n = 10$), determined applying the *R1* gate on CD90 vs. CD31 dot-plot (green box in **Figure 2A.3**), and were then processed for subsequent culture. Otherwise, two MPC primary cultures revealed purity lower than 95% (data not shown) with a consistent percentage (10 and 8%) of MSC-like cells, determined applying the *R2* gate (blue box in **Figure 2A.3**). These latest two cell preparations were excluded from the study.

Specific Formulated Media Induced Differentiation of MPCs

In order to verify the differentiation potential of the isolated MPCs, two passages of mesengenic differentiation were applied. After 2 weeks of culture in MesenPRO® RS medium, differentiated cells (P2-MSCs) showed a fibroblastoid and spindle-shaped morphology, typical of MSC-like cells (**Figure 2B.1**). F-actin resulted re-organized in stress-fibers (red in **Figure 2B.2**), while very low levels of nestin expression was reported in few rare cells. Flow cytometry revealed almost homogeneous culture of CD90-positive cells, lacking MPC-related markers as CD31, CD18, and CD11c (**Figure 2B.3**). As a consequence, in the CD90 vs. CD31 dot-plot these cell events were plotted in the *R2* gate. In order to definitely confirm the MSC nature of the differentiated cells, P2-MSC cultures were exposed to adipogenic as well as osteogenic differentiating conditions, and respectively the lipid droplet accumulation and calcium deposition were report for the cultures investigated ($n = 10$, data not shown).

MPCs Interact with NGs

Initial cell attachment on T2 nanostructures was not significantly altered. After the 4 h incubation, most of the seeded cells resulted firmly adherent to the substrate surfaces without any noticeable difference with respect to those on FLAT or CTRL. After overnight incubation, MPC morphology resulted highly conserved, with cells maintaining the typical rounded fried egg-shape. Nuclei appeared slightly oval with compact chromatin, and did not show any alignment to the grating direction. Conversely, all characteristic podosome-like structures resulted aligned in correspondence to the NG crests, while resulting randomly oriented in correspondence of the not patterned areas, similarly to those of the cells seeded on FLAT or CTRL (**Figure 3**). Moreover, numerous filopodia, occasionally detected on some MPCs, showed the ability to align to the NGs.

Nanogratings Trigger MPC Differentiation toward MSC-Like Cells

In order to evaluate the effect of the solely topography of the T2 on the MPCs transition to MSCs, the cell mortally related to the re-plating procedures or to the different substrate chemical

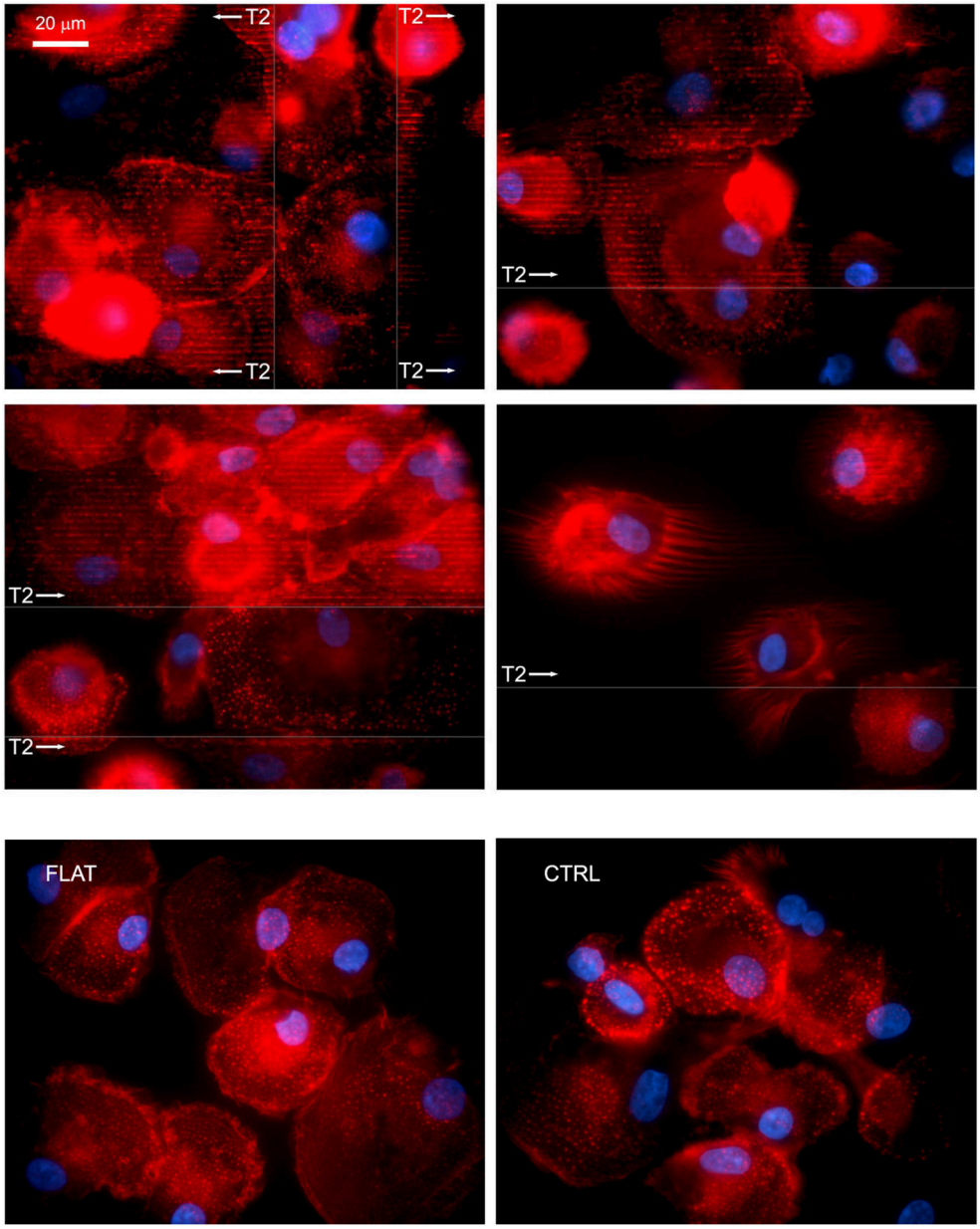

FIGURE 3 | MPCs interact with NGs at the adhesion level. After overnight incubation on nano-structured T2 gratings, most of the seeded MPCs resulted firmly attached and showed the numerous podosomes aligned to the grating direction (white arrows) and in correspondence to the crests. Similarly, filopodia showed an alignment to the grating direction. Fluorescence images were acquired by confocal microscopy and immunostaining of F-Actin; arrows show grating direction, and area. The bottom row shows cells on FLAT (left) and CTRL (right).

composition have been taken in consideration, eliminating the experiments that showed reduced vitality (<80%) in CTRL and in FLAT cultures (**Figure 4A** pale red area). For the same reason, possible spontaneous differentiation has been evaluated, and experiments in which CTRL or FLAT cultures showed a MSC/MPC ratio higher than 0.1 where excluded from the study (**Figure 4B**). After this evaluation eight experiments showed consistent vitality and absence of spontaneous differentiation (**Figure 4C**), while two experiments were excluded from the analysis (**Figure 4D**). After 7 days of culture on FLAT inserts, no change in MPC morphology or phenotype were reported and few events were detected in the *R2* gate, in all validated experiments (**Figure 5A**). Conversely on T2 NGs, evident signs of differentiation were proven by numerous elongated MSC-like cells oriented along the grating direction, and by a consistent population expressing MSC-associated phenotype, detected in the *R2* gate (**Figure 5B**). Quantitatively, normalized MSC/MPC ratio resulted significantly higher in cultures performed on T2

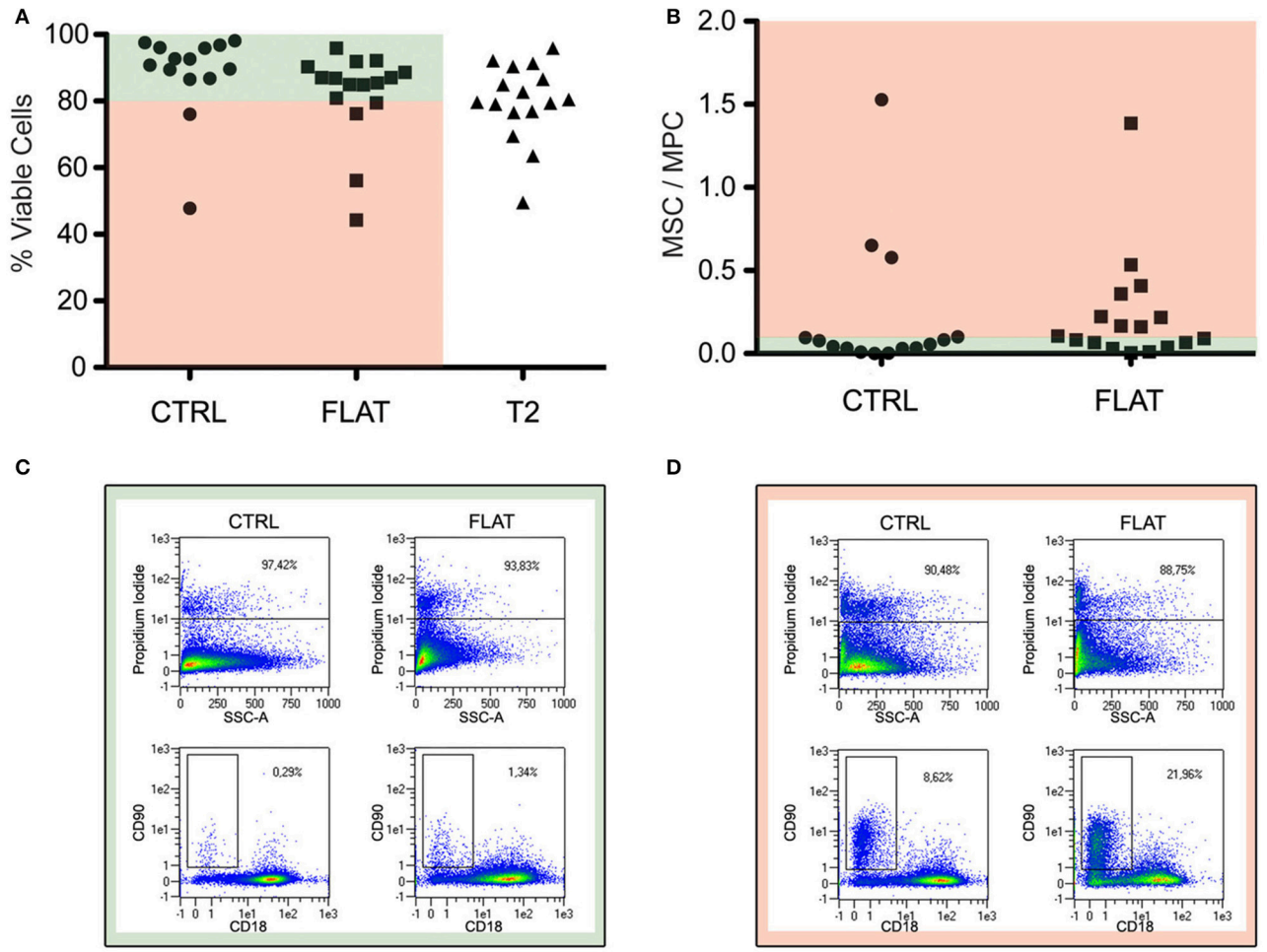

FIGURE 4 | Evaluation of cell viability and spontaneous differentiation. (A) Percentage of viable cells (PI-negative) for the different substrates. **(B)** Spontaneous differentiation, evaluated by ratio between MSC and MPC percentages, in control sub-cultures and "FLAT" conditions. Red and green areas indicate data from experiments that were considered valid (i.e., viability >80% and MSC/MPC <0.10 on controls). **(C)** Dot plots of representative sample included in the study. **(D)** Dot plots of representative samples excluded from the study for its consistent spontaneous differentiation in non-nanostrucutred controls.

NGs ($p < 0.05$), with the mean value (0.19 ± 0.05) triplicated with respect to that measured on FLAT (0.062 ± 0.015, **Figure 5C**).

DISCUSSION

Implant osteointegration represents the key of success in most of the orthopedic procedures. It has been defined as the formation of structural and functional interconnection between the implant and the host bone (Albrektsson et al., 1981). In order to promote osteointegration, many materials and new fabrication processes have been proposed to obtain a high porous implant surface on which the bone cells could adhere and growth (Agarwal and García, 2015). Nowadays, the gold standard is represented by devices made by trabecular metal, which can mimic the bone microstructure and make available an ideal space for cellular colonization and revascularization of the neoformed bone tissue. However, an important limit of the implants and materials currently used in orthopedic surgery is that they present micron-sized textures while the bone is a nanostructured tissue. Collagen and hydroxyapatite, for example, provide a unique nanostructured scaffold for proteins and bone cell interactions. For these reasons, nanomaterials have been proposed with the aim of improving surface properties and creating an environment more conducive for osteoblast function and bone ingrowth (Tran and Webster, 2009; Parchi et al., 2013). Sub-micrometer sized features on traditional implants can control protein absorption and decrease scar tissue growth, bacterial infection and promote appropriate tissue growth. Moreover, they can also guide cell differentiation through physical stimulation, by a process called mechanotrasduction in which physical forces are converted into biochemical signals that are the finally integrated to give specific cellular response (Duncan and Turner, 1995).

Substrate nanotopography has been demonstrated to affect the biology of MSCs *in vitro* and the possibility of controlling the phenotype of these cells by just physically modifying the substrate topography represents one of the most promising

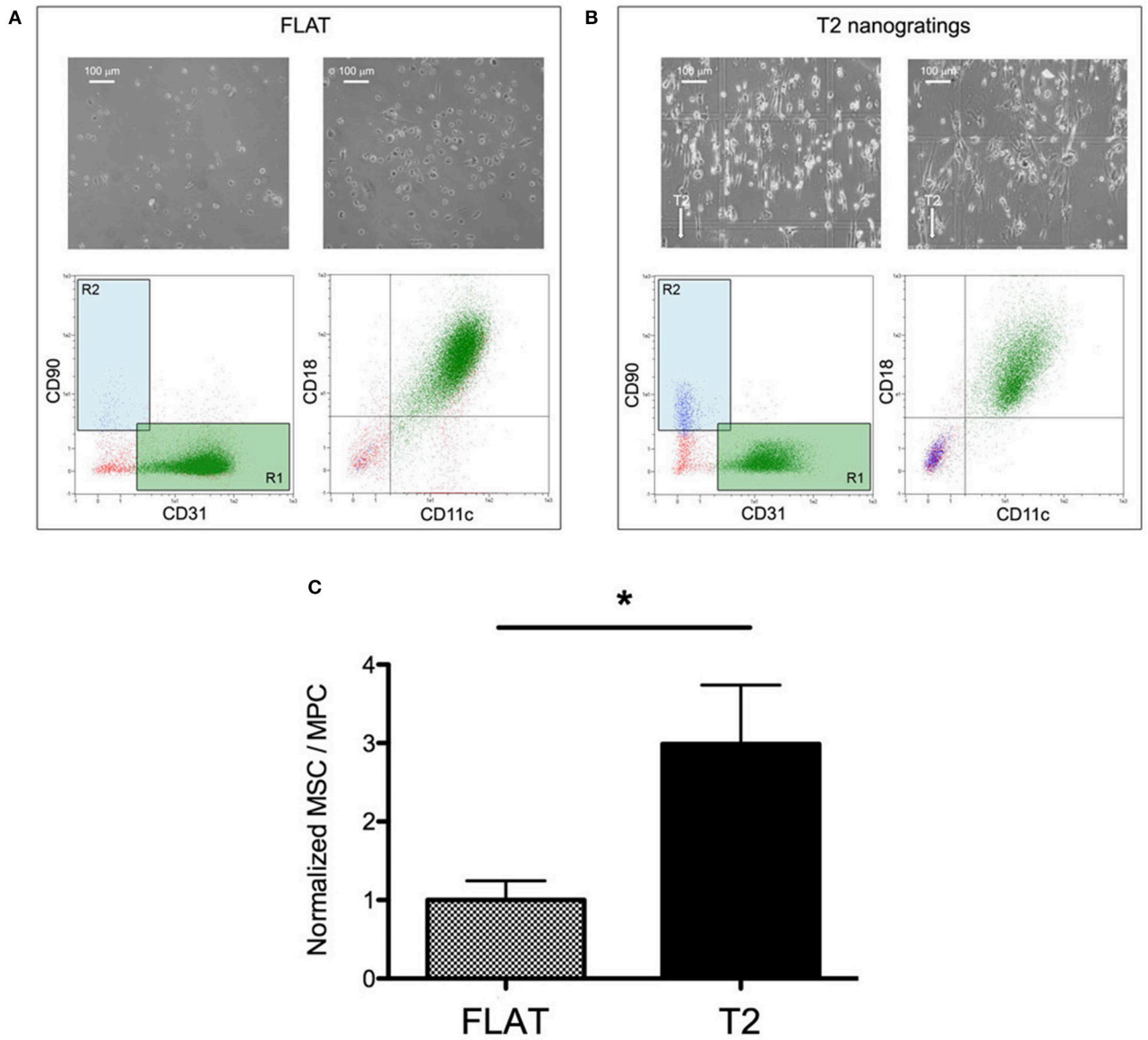

FIGURE 5 | Nanograting induced MPC-to-MSC differentiation. Representative flow-cytometry scatter plots and microscopy images of MPCs cultured on Flat **(A)** and T2 **(B)**. **(C)** Ratio of viable MSCs over viable MPCs normalized to the average value measured on Flat (*$p < 0.05$, *t*-test).

innovations in the field. However, the majority of the studies was conducted with bone marrow derived mesenchymal stromal cells (BM-MSCs). Very recently our group demonstrated that nanogratings, alternate lines of sub-micrometer sized ridges and grooves, can effectively polarize BM-MSCs by pure contact interaction (Antonini et al., 2014), and promote enhanced osteo-differentiation with respect to flat surfaces (Antonini et al., 2016). There is indeed a large consensus considering BM-MSCs as the progenitors of the skeletal tissue related cell lineages for their ability to differentiate into adipocytes, osteoblasts and chondroblasts (Keating, 2012). This study also shows that after interaction with nanograting, BM-MSCs retain their osteodifferentiation potential.

Nonetheless, BM-MSCs represent a heterogeneous cell population isolated *in vitro*, in which the composition and cell biology are strongly affected by the isolating and expanding procedures (Phinney, 2012). Alongside the variability related to donors (Deasy et al., 2007; Siegel et al., 2013) and culture methods (Sharma et al., 2014), multiple origins of BM-MSCs were hypothesized and supported by several experiments demonstrating that genuine MSC cultures can be obtained by different BM subpopulations *in vivo* and *in vitro,* (reviewed in Pacini, 2014).

Remarkably, the MSC frequency in human normal bone marrow has been estimated only about the 0.001 and 0.01% of the mononuclear cells. Conversely, the MPCs frequency, and its

in vivo counterpart denominated Pop#8, results from two to three logs higher (Trombi et al., 2009; Pacini et al., 2016).

Therefore, it is reasonable to hypothesize that the effects exerted by the nanostructured surfaces on MPCs can more strongly affect implant osteointegration than those on very small cell populations such us "skeletal stem cells" (SSCs) (Bianco and Robey, 2015) or $CD271^{+}CD140^{low/-}$ (Li et al., 2016), today considered the *bona fide* ancestors of BM-MSCs *in vivo*. Our results indicate that nanogratings can promote MPC to MSC transition, suggesting an *in vivo* scenario in which osteointegration is possibly promoted following MPC to MSC transition and nanograting-driven MSC osteogenic differentiation.

It is known that the substrate directionality stimulus is optimally delivered to many kind of undifferenziated cells, including MSCs, which in turn elongate and align to the nanograting lines. This polarization occurs also at level of cytoskeleton fibers and, though to a lesser extent, of nuclei. In this context, an elegant mechanistic model was proposed suggesting that an increased binding of integrins to ECM proteins would lead to increased FAK recruitment to the adhesion plaque inducing downstream ERK-dependent differentiation (Yee et al., 2008) This model provides an insight into the mechanisms of focal adhesion-dependent differentiation that might apply also to the MPC-MSC transition, and shows that nanotopographical surface modifications may directly regulate stem cell differentiation. Substrate topography can, indeed, interfere with focal-adhesion maturation and shaping, which in turn reflects on cellular mechanical stress distribution and shaping.

Interestingly, here we have demonstrated that the MPC mesengenic differentiation could be induced by the solely interaction with the T2 NGs, in absence of any other external stimuli. This aspect opens an interesting issue regarding the heterogeneity of culture expanded MSCs. In fact, it has been hypothesized that expanding MSCs from unfractionated "crude" bone marrow cell suspensions, in uncontrolled open culture systems, could lead to significantly different cell products with unpredictable biological properties because of mild modifications in the culture determinants or even to environmental fluctuations during cell expansion (Pacini, 2014). Data presented here suggest that differences in the intrinsic nanotopography of the culture surfaces, correlated to the materials or to the production processes, should be consider an additional culture determinant that might increase the variability of the final product. Thus, it might be possible that some pre-clinical data collected on MSCs expanded in flasks from bone marrow are not predictive for the therapeutic value of the same cell population cultured in bioreactors (i.e., equipped with hollow fibers), as their proliferation and differentiation potential could be altered by the specific micro- and/or nanotopography of the contact surfaces.

Concluding, the seminal findings reported here sustain the fascinating hypothesis of a topographic control of progenitor cells fate by specific designs of nanostructured surfaces. This aspect is of particular interest in the case of multipotent progenitors as MPCs that, retaining both mesengenic and angiogenic potential, possess a high clinical appeal.

AUTHOR CONTRIBUTIONS

SA and MM: Conception and design, Data collection, assembly, analysis, and interpretation; EJ: Data collection, assembly, analysis, and interpretation; SM: Microfabrication of nanostrucutres; PP, SB, FP: Data collection, assembly, analysis, and interpretation; SP: Conception and design, Data collection, assembly, analysis, and interpretation. Manuscript writing; IP: Manuscript writing. MC: Conception and design, Data collection, assembly, analysis, and interpretation. Manuscript writing.

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Glycan Profiling Shows Unvaried *N*-Glycomes in MSC Clones with Distinct Differentiation Potentials

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Different cell types have different *N*-glycomes in mammals. This means that cellular differentiation is accompanied by changes in the *N*-glycan profile. Yet when the *N*-glycomes of cell types with differing fates diverge is unclear. We have investigated the *N*-glycan profiles of two different clonal populations of mesenchymal stromal cells (MSCs). One clone (Y101), when differentiated into osteoblasts, showed a marked shift in the glycan profile toward a higher abundance of complex *N*-glycans and more core fucosylation. Yet chemical inhibition of complex glycan formation during osteogenic differentiation did not prevent the formation of functional osteoblasts. However, the *N*-glycan profile of another MSC clone (Y202), which cannot differentiate into osteoblasts, was not significantly different from that of the clone that can. Interestingly, incubation of Y202 cells in osteogenic medium caused a similar reduction of oligomannose glycan content in this non-differentiating cell line. Our analysis implies that the *N*-glycome changes seen upon differentiation do not have direct functional links to the differentiation process. Thus *N*-glycans may instead be important for self-renewal rather than for cell fate determination.

Keywords: MALDI-MS, *N*-glycan, FANGS, immunomodulatory MSCs, multi-lineage differentiation, self-renewal

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INTRODUCTION

Mesenchymal stromal cells (MSCs) are a heterogeneous population of cells that contains both adult multipotent and immunomodulatory cell types (Nauta and Fibbe, 2007). They can be isolated from several locations in the human body by exploiting their ability to adhere to plastic. MSCs are often distinguished from other cells types by the expression of CD105, CD73, CD90 cell surface markers and the absence of hematopoietic markers such as CD45, CD43, CD14, and CD19 (Dominici et al., 2006). Once isolated, MSCs can be induced to differentiate into bone, cartilage or fat cells, but not all the cells in a heterogeneous primary isolate behave the same way during a differentiation experiment. In fact, clonal sub-populations of primary MSCs show different features than the parent population. Some clones can differentiate into all three lineages. Others are only capable of dual or single lineage differentiation, while some do not differentiate at all (Pittenger et al., 1999). In addition, MSCs have been observed to provide immunomodulatory functions, a role that is shared by both differentiating and non-differentiating cells (Bartholomew et al., 2002; James et al., 2015). Given the range of phenotypic characteristics and the lack of decisive markers, the precise identification of MSCs is not trivial in a primary mixture. Moreover, the characterization of different MSC lines derived from primary cells would also benefit from the elucidation of further defining molecular features.

We generated single cell-derived clones from immortalized bone-marrow MSCs. Importantly, different clones obtained in the course of immortalization represent the various cell types contained in the heterogeneous primary mixture. Four such clones have been characterized in detail, two of which are capable of differentiation, while the other two represent MSCs with enhanced immunomodulatory features that cannot differentiate (James et al., 2015). The two differentiating lines (Y101 and Y201) are similar in their gene expression profiles, and both show tri-lineage differentiation potential, although Y101 cells are primed for osteogenic differentiation, and do not differentiate efficiently into adipocytes. The non-differentiating lines (Y102 and Y202) exhibit little differentiation capacity with possibly a very weak adipogenic potential, but express a significant proportion of pro-inflammatory genes, with increased IL-7 production. They are characterized by the unique cell-surface marker CD317, and represent approximately 10% of the mixed primary MSC population. Apart from the inflammatory markers, there are as yet no clear differences in the four lines that would indicate why the '01 lines can and the '02 lines cannot differentiate. These MSC lines thus represent excellent models to study the molecular characteristics of the various MSC populations found *in vivo*.

Cellular differentiation is accompanied by a change in glycosylation patterns. For example, the antigens marking different stages of embryonic stem cell development are glycans (Gooi et al., 1981). Along similar lines, the *N*-glycome undergoes remodeling during embryonic cell differentiation (Amano et al., 2010). Glycosylation differences also exist between cells that are at the same stage of differentiation, but have different lineage commitments, such as the extensive differences observed in lectin binding affinities for neuronal and mesenchymal progenitor cells (Dodla et al., 2011). The differentiation of primary MSCs also leads to glycosylation changes. Glycomic comparison of adipocytes and their parental MSCs (a heterogeneous primary population) has uncovered increased sialylation and decreased branching of complex *N*-glycans as a consequence of differentiation (Hamouda et al., 2013). In contrast, osteogenic differentiation of another heterogeneous primary cell population has been associated with a decrease in the levels of oligomannose type *N*-glycans (Heiskanen et al., 2009). Although many other changes were observed between MSCs and the derived osteoblasts, detailed quantification of the differences is skewed by the fact that glycan permethylation was not used prior to MALDI-MS analysis, which prevents the quantification of relative glycan abundances (Wada et al., 2007).

While glycosylation differences are a well-accepted feature of different cell types, the functional significance of these variations is less clear. Moreover, it is unknown whether MSC sub-types with different properties, such as differentiation-competent MSCs vs. primarily immunomodulatory MSCs, have differences in their glycosylation profiles. We therefore asked if differences in the glycosylation state between the various MSC sub-populations in our model cultures could be observed, and could potentially contribute to phenotypic differences between the MSC subtypes within a primary mixture. In accordance with previous findings (Heiskanen et al., 2009), we observed a robust change in the *N*-glycan profile during osteogenesis of the tested homogeneous MSC line. Interestingly, detailed quantitative *N*-glycan profiling showed remarkable similarity for two different immortalized MSC lines (Y101 and Y202) despite their differing phenotypes.

Abbreviations: ALP, Alkaline phosphatase; FANGS, Filter-aided *N*-glycan separation; MALDI, Matrix-assisted laser desorption/ionization; MS, Mass spectrometry; MSC, Mesenchymal stromal cell; SEM, Standard error of the mean; TOF, Time-of-flight.

MATERIALS AND METHODS

Cell Culture and Osteogenic Differentiation

Cells were cultured in basal medium composed of DMEM (high glucose, pyruvate, no glutamine) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% GlutaMax-I. To stimulate osteogenesis, cells were seeded at a density of 20,000 cells/cm^2 and 50 μg/mL ascorbic acid, 5 mM β-glycerophosphate, and 10 nM dexamethasone were added to basal medium. Culture medium was changed every 3–4 days during assays. For histochemistry cells were cultured in 24-well dishes, for glycan profiling in 10 cm plates.

Preparation of *N*-glycans for Mass Spectrometry

The filter-aided *N*-glycan separation (FANGS) method was carried out as described (Abdul Rahman et al., 2014). Briefly, 1×10^6 cells were seeded into a 10 cm culture dish and harvested 24 h later for glycan preparation. Following SDS lysis and buffer exchange in a centrifugal filter, glycans were released using PNGase F treatment, and permethylated prior to mass spectrometric analysis.

Mass Spectrometry

Permethylated glycans were dissolved in 20 μL methanol. Two microliters of this sample was mixed with 1 μL of 0.5 M sodium nitrate (in 70% methanol) and 2 μL of 20 mg/mL 2,5-dihydroxybenzoic acid (in 70% methanol). Two microliters of this mix was spotted onto a ground steel MALDI target plate (Bruker) and allowed to air dry. Immediately afterwards, 0.2 μL of ethanol was added to the spot and left to air dry for re-crystallization. Samples were analyzed using an ultraflex III MALDI-TOF mass spectrometer (Bruker). The spectra were acquired over the *m/z* range 800–6000 using positive-ion mode, with 4000 laser shots in steps of 800, which were summed to give one spectrum per spot. The Smartbeam™ laser power was set to 50–65%.

Data Analysis

Spectra were analyzed using flexAnalysis 3.3 (Bruker), first processed using the centroid peak detection algorithm, using a signal to noise limit of 3, and smoothed for 1 cycle at 0.1 *m/z*, using the Savitzky-Golay algorithm. Glycan peaks were identified from their mono-isotopic peak *m/z* values on comparison with those for established *N*-glycan structures. For a glycan to be included in the analysis at least two of its isotopic peaks had to have intensities >3x the noise, and the associated peaks had to fall into an isotopic pattern as predicted based on the glycan's atomic

composition (Bruker Compass's Isotope Pattern function). The peak intensities of all isotope signals >3x noise for a given glycan were summed to provide that glycan's total peak intensity. To compare different spectra, the total peak intensity values were normalized to the total peak intensities of all glycans identified in that spectrum. Normalized peak intensities could then be averaged across different spectra of the same sample type.

Flow Cytometry Analysis

Cells were washed twice with PBS, and incubated with washing buffer (0.2% bovine serum albumin, 5 mM EDTA in PBS) at 37°C until cells detached. Cells were centrifuged for 5 min at 450 g, resuspended in PBS and 150,000 cells pelleted and resuspended in 120 μL ice-cold PBS. Following 15 min incubation on ice, 120 μL of 10 μg/mL FITC-ConA (Vector Labs) was added to each sample. After two 15 min incubations interspersed with flicking of the tubes, 1 mL of washing buffer was added and the samples were centrifuged for 5 min at 450 g. The cell pellet was resuspended in 100 μL of washing buffer containing 1 μg/mL DAPI and incubated on ice in the dark for 5 min, followed by the addition of 1 mL washing buffer, and centrifugation for 5 min at 450 g. The pellet was resuspended in 400 μL PBS for flow cytometry. Data were gated for DAPI-negative FITC-positive cells. Median fluorescence intensity was determined for all gated cells in the sample.

Analysis of cell surface markers was performed as previously described (James et al., 2015).

Statistics

Data were analyzed using Sigma Plot 12.3. Before statistical tests were applied, a normality test (Shapiro–Wilk) and a test of equal variance was performed. For comparing two groups if data passed, a Student's *T*-test was carried out. Otherwise, one-way ANOVA tests were carried out, followed by Holm Sidak *post-hoc* tests if required. If the data failed the normality or variance tests, equivalent non-parametric tests were applied instead. Either a Mann–Whitney test when two groups were being compared, or a Kruskal–Wallis one-way ANOVA on ranks, followed by Dunn's *post-hoc* tests was carried out if more than one group was being compared. Throughout $^{*}: P < 0.05$, $^{**}: P < 0.01$, and $^{***}: P < 0.001$.

RESULTS

Osteogenic Differentiation of Y101 Cells Alters Their *N*-glycan Profile

To study the sub-type-specific properties of cells from a primary human MSC mixture, previously generated telomerase immortalized lines derived from primary human MSCs, termed hTERT-MSCs (James et al., 2015), were used. The *N*-glycans of one of these lines, Y101, were profiled using the FANGS method (Abdul Rahman et al., 2014). **Figure 1** shows a typical *N*-glycan profile of Y101 cells in which as many as 65 different glycan species were detected. The glycan profile is qualitatively comparable with those reported for primary MSCs (Heiskanen et al., 2009; Hamouda et al., 2013), although different glycan classes cannot be quantitatively compared with those reported in the older studies, due to their different analysis strategies. The most abundant glycans we observe in these cells are the Man_6-Man_9 oligomannose species (*m/z* 1783.85, 1987.93, 2192.03, and 2396.14). Among the complex glycans, the singly sialylated, fucosylated biantennary species (*m/z* 2605.24), followed closely by the same glycan without sialic acid (*m/z* 2244.08) and then the bis-sialylated version (*m/z* 2966.42) are most abundant. It is worth noting that the majority of the large complex glycans are fucosylated (e.g., *m/z* 4226.04, 4314.14, 4587.21, 4676.28, 4764.41, 5037.41, and 5124.57), which most likely indicates the presence of core fucose.

Changes in the *N*-glycan profile of primary MSCs upon differentiation have been reported (Heiskanen et al., 2009), again with the caveat that that analysis did not allow detailed quantification. The telomerase-immortalized Y101 cell line is capable of robust osteogenic differentiation *in vitro* (James et al., 2015). This is demonstrated when the cells are stained for secreted alkaline phosphatase (ALP, pink staining) and hydroxyapatite mineral deposits (brown) with an ALP/von Kossa stain at 7 day intervals over a 3 week period (**Figure 2A**). It is clear from appearance of the pink ALP stain and the stained phosphate deposits that 21 days in osteogenic medium gives strong differentiation (**Figure 2A** bottom row "21 days" image). These differentiated cells were harvested and their total *N*-glycan repertoire analyzed using FANGS followed by MALDI-TOF-MS (**Figure 2B**). The resulting spectra show a very different picture than that from the undifferentiated MSCs. Importantly, the observed changes in glycosylation during osteogenesis were not due to the 21 days spent in culture. When Y101 MSCs were cultured in basal rather than osteogenic medium for 21 days (a treatment that does not promote osteogenesis) the *N*-glycan spectra obtained were similar to those from MSCs at the start of the incubation period (**Figures 2E,F** and **Supplementary Figure 1**). In the differentiated osteoblasts, by far the most abundant glycan peak is now the singly sialylated, fucosylated biantennary complex glycan species (*m/z* 2605.22), followed, at about half the intensity, by the same complex glycan without sialic acid (*m/z* 2244.08) and the fully sialylated species (*m/z* 2966.39). The most abundant oligomannose glycan in osteoblasts, the Man_6 species (*m/z* 1783.87), is only ranked fourth in relative intensity in the *N*-glycome of osteoblasts (**Figure 2B**, **Table 1**).

Averaging the glycan peak intensities from several biological replicates showed that the most abundant complex glycans were indeed reproducibly much more abundant in osteoblasts than undifferentiated MSCs (**Figure 2C**, **Table 1**). In contrast, the large oligomannose glycans all had very low abundances in the *N*-glycomes of osteoblasts, although these are among the most abundant glycans in those of the MSCs (**Figure 2C**, **Table 1**). Similarly, the singly glucosylated oligomannose glycan species was undetectable from osteoblasts, although it was notably present from MSCs (*m/z* 2600.22 **Figures 1**, **2D**, **Table 1**). There was also a trend toward increased abundance of hybrid glycans in osteoblasts (**Figure 2D**, **Table 1**). Differences are even more evident when the total oligomannose and complex glycan levels are compared. The reduction in relative levels of oligomannose glycans and the increase in complex glycans are both highly

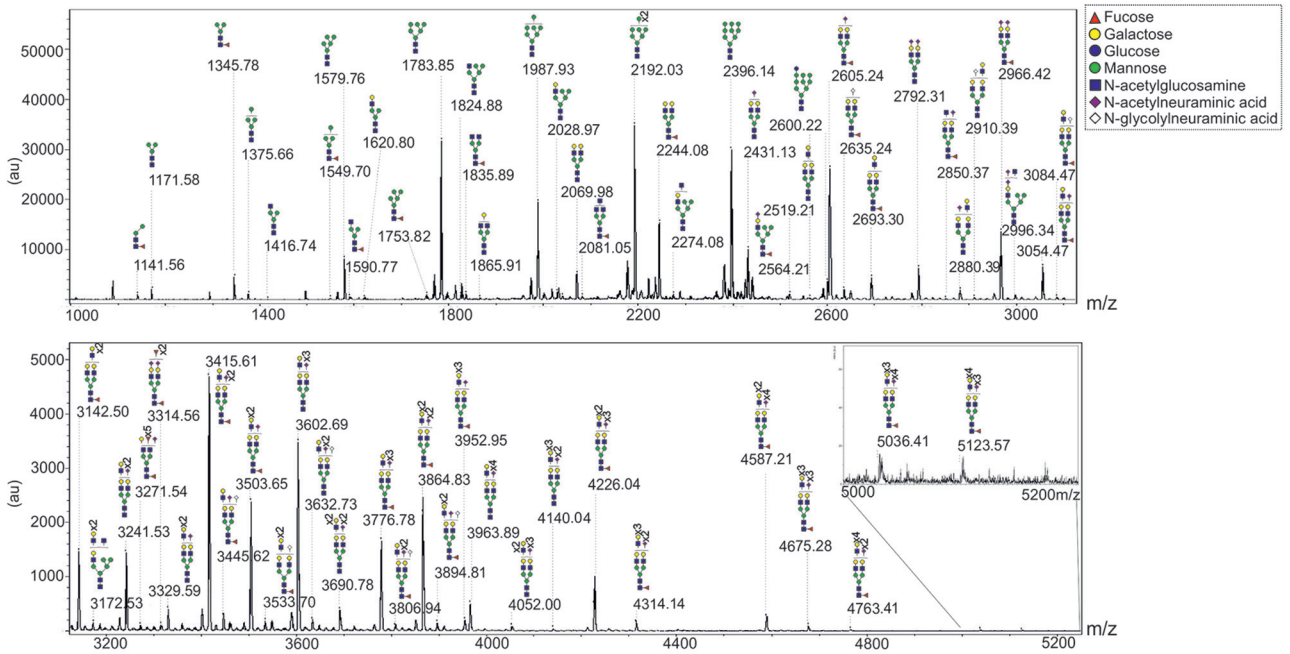

FIGURE 1 | *N*-glycan profile of Y101 hTERT-MSC line. Representative MALDI-TOF spectrum of permethylated *N*-glycans isolated from the Y101 hTERT-MSC line. Peaks were annotated based on the calculated glycan mass and knowledge of the glycan biosynthesis pathway. *m/z* value of the mono-isotopic peak is labeled, with the predicted structure pictured above. Spectrum shown is from *m/z* 1000 to 5250 split into two sections. Note that the sections are plotted on different y axis scales to make the higher *m/z* range section easier to see.

significant (**Figure 2E**). There is also a significant increase in the total amount of fucosylated species detected from the osteoblasts over that from their MSC progenitors (**Figure 2F**), although glycan species with multiple fucoses were more prevalent in the MSCs (**Table 1**).

MSC Lines Share a Similar *N*-glycan Profile Independent of Their Altered Differentiation Potentials

Given that differentiation considerably alters the *N*-glycan profile, we wondered whether MSCs with diverse differentiation abilities present variations in *N*-glycan profiles. We compared two hTERT-MSC lines: Y101 and Y202. Y101 cells differentiate well into osteoblasts. Y202 cells in contrast, which do show the cell surface markers characteristic of MSCs (**Supplementary Figure 3**; Dominici et al., 2006), are virtually devoid of differentiation potential (James et al., 2015) as exemplified by the lack of ALP and von Kossa staining, used to detect osteogenesis (**Figure 3A** bottom row).

To assess if the differences in differentiation capacity between the different clonal MSC lines are associated with a variation in their *N*-glycan repertoire, we used FANGS followed by MALDI-TOF-MS to profile their glycans. Both MSC lines were cultured for 24 h in basal medium post passaging, prior to *N*-glycan sample harvest and analysis. The overall *N*-glycan profiles of these lines look very similar to each other (**Figure 3B**). Profiling of several biological replicates for the same cell lines followed by averaging of the relative glycan abundances revealed no significant differences at all (**Supplementary Figures 2A,B** and **Supplementary Table 1**). This lack of difference is also apparent when comparing different glycan classes (**Supplementary Figures 2C,D**). A heat map generated using the averaged relative abundances of all glycans from the two different MSC lines, Y101 and Y202, and the osteoblasts derived from Y101 cells clearly shows that the main change in *N*-glycan profile is between the MSCs and the differentiated progeny, rather than within the different lines of MSCs (**Figure 3C**).

It is clear that differentiation alters the glycan profile, yet the glycan profile of the stem cells does not provide clues to their differentiation potential. We therefore wondered if the glycosylation changes are restricted to cells undergoing differentiation, or whether non-differentiating cells, when incubated under differentiation-promoting conditions, alter their glycan profiles. The reduction of oligomannose glycan content was investigated for MSCs incubated in osteogenic medium for 8 days. As expected, Y101 cells showed a clear reduction in oligomannose content, as evidenced by reduced ConA-FITC staining measured using flow cytometry when compared with cells cultured in basal medium (**Figure 3D**, second and third bars). The lectin ConA binds oligomannose glycans, and is therefore a sensitive tool for uncovering changes in the proportion of this glycan class. Surprisingly, Y202 cells, which do not form mineral deposits, showed the same highly significant reduction in oligomannose content when incubated in osteogenic

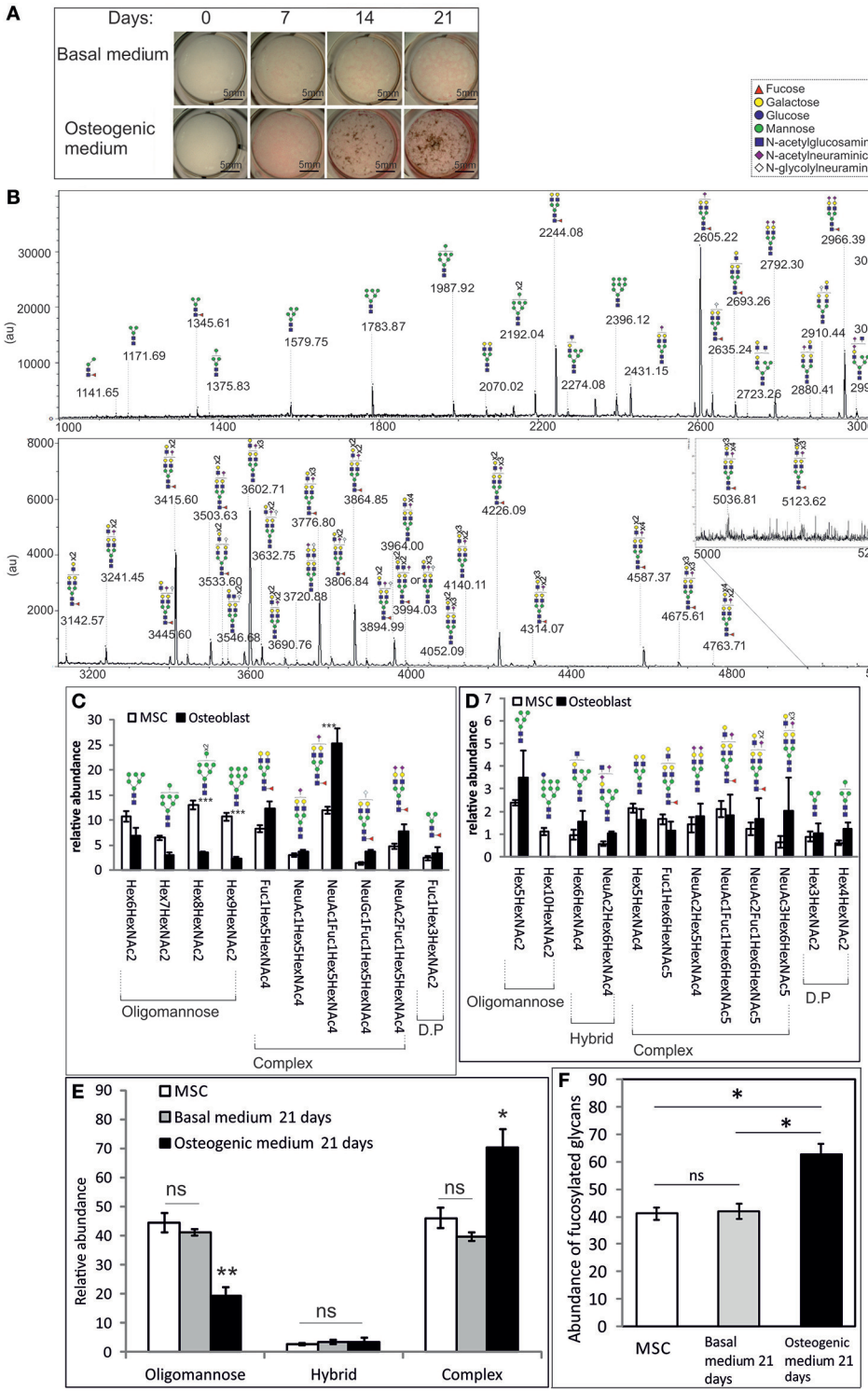

FIGURE 2 | Osteogenic differentiation significantly alters the *N*-glycan profile of MSCs. (A) Brightfield images of Y101 hTERT-MSCs cultured in basal **(top row)** or osteogenic **(bottom row)** media, stained for alkaline phosphatase (pink) and phosphate (von Kossa, brown), sampled at weekly intervals. **(B)** A representative MALDI-TOF spectrum of permethylated *N*-glycans isolated from osteoblasts cultured for 21 days as in **(A)**. Presented as in **Figure 1**. **(C–F)** Comparisons of averaged (Y101 $n = 5$, osteoblast $n = 3$) normalized total peak intensities of: **(C)** Individual glycan structures with abundances above 3% of the total. **(D)** Individual glycan structures with abundances of 1–3% of the total. D.P, degradation product, most likely produced in the lysosome. **(E)** Sums of different glycan types. **(F)** Sums of all fucosylated glycan abundances. Error bars show standard error of the mean. In **(E, F)** the "Basal medium 21 days" sample was harvested following culture in basal medium for 21 days ($n = 2$). For a representative spectrum of this sample see **Supplementary Figure 1**. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

TABLE 1 | Averaged relative abundances of individual *N*-glycan structures isolated from Y101 hTERT-MSCs and their osteoblast progeny as well as MSCs kept for 21 days in basal medium.

Glycan composition	Type[a]	Average relative abundance[b]		
		MSC	Osteoblast	21 day basal
$Hex_5HexNAc_2$	O	2.394 ± 0.120	3.505 ± 1.182	7.095 ± 1.526
$Fuc_1Hex_5HexNAc_2$	O	0.621 ± 0.215	0.187 ± 0.187	0.885 ± 0.405
$Hex_6HexNAc_2$	O	10.740 ± 0.998	6.846 ± 1.639	13.342 ± 2.521
$Hex_7HexNAc_2$	O	6.470 ± 0.480	3.010 ± 0.499	6.258 ± 1.252
$Hex_8HexNAc_2$	O	13.042 ± 0.894	3.476 ± 0.160	9.089 ± 0.790
$Hex_9HexNAc_2$	O	10.693 ± 0.825	2.383 ± 0.345	5.174 ± 0.521
$Hex_{10}HexNAc_2$	O	1.119 ± 0.154	0	0.186 ± 0.062
$Hex_5HexNAc_3$	H	0.584 ± 0.114	0.509 ± 0.328	0.615 ± 0.166
$Hex_6HexNAc_3$	H	0.212 ± 0.130	0.638 ± 0.370	0.916 ± 0.192
$Hex_6HexNAc_4$	H	0.965 ± 0.217	1.557 ± 0.483	1.246 ± 0.351
$NeuAc_1Hex_6HexNAc_3$	H	0.432 ± 0.184	0.594 ± 0.302	0.709 ± 0.090
$NeuAc_1Fuc_1Hex_6HexNAc_3$	H	0.032 ± 0.032	0	0
$Hex_7HexNAc_5$	H	0.438 ± 0.127	0.207 ± 0.089	0.125 ± 0.040
$NeuAc_2Hex_6HexNAc_4$	H	0.581 ± 0.108	1.032 ± 0.084	0.304 ± 0.269
$Hex_8HexNAc_6$	H	0.066 ± 0.031	0	0
$Hex_3HexNAc_3$	C	0.004 ± 0.004	0.178 ± 0.178	0.091 ± 0.112
$Fuc_1Hex_3HexNAc_3$	C	0.431 ± 0.084	0.185 ± 0.185	0.292 ± 0.195
$Hex_4HexNAc_3$	C	0.335 ± 0.085	0.197 ± 0.197	0.323 ± 0.112
$Fuc_1Hex_3HexNAc_4$	C	0.686 ± 0.106	0.126 ± 0.126	0.287 ± 0.101
$Hex_4HexNAc_4$	C	0.285 ± 0.056	0.089 ± 0.089	0.224 ± 0.134
$Fuc_1Hex_4HexNAc_4$	C	0.552 ± 0.119	0	0
$Hex_5HexNAc_4$	C	2.151 ± 0.214	1.622 ± 0.485	3.370 ± 0.447
$Fuc_1Hex_3HexNAc_5$	C	0.297 ± 0.093	0	0.000 ± 0.138
$NeuAc_1Fuc_1Hex_4HexNAc_3$	C	0.379 ± 0.104	0.139 ± 0.139	0.597 ± 0.269
$Fuc_1Hex_5HexNAc_4$	C	8.259 ± 0.655	12.271 ± 1.386	11.951 ± 2.082
$NeuAc_1Fuc_1Hex_5HexNAc_3$	C	0	0.138 ± 0.138	0.337 ± 0.132
$NeuAc_1Hex_5HexNAc_4$	C	3.040 ± 0.347	3.757 ± 0.367	3.330 ± 0.169
$Hex_6HexNAc_5$	C	0.383 ± 0.123	0.088 ± 0.088	0.204 ± 0.045
$NeuAc_1Fuc_1Hex_5HexNAc_4$	C	11.968 ± 0.722	25.387 ± 2.830	12.152 ± 4.007
$NeuGc_1Fuc_1Hex_5HexNAc_4$	C	1.348 ± 0.273	3.712 ± 0.423	1.346 ± 0.789
$Fuc_1Hex_6HexNAc_5$	C	1.661 ± 0.213	1.171 ± 0.382	0.590 ± 0.184
$NeuAc_2Hex_5HexNAc_4$	C	1.421 ± 0.340	1.808 ± 0.547	0.619 ± 0.078
$NeuAc_1Fuc_1Hex_5HexNAc_5$	C	0.143 ± 0.059	0	0
$NeuAc_1Hex_6HexNAc_5$	C	0.461 ± 0.101	0.314 ± 0.150	0.125 ± 0.033
$NeuGc_1Hex_6HexNAc_5$	C	0.252 ± 0.052	0.140 ± 0.112	0.040 ± 0.032
$NeuAc_2Fuc_1Hex_5HexNAc_4$	C	4.784 ± 0.583	7.695 ± 1.401	2.957 ± 1.398
$NeuAc_1Fuc_1Hex_6HexNAc_5$	C	2.102 ± 0.360	1.827 ± 0.904	0.599 ± 0.323
$NeuGc_1Fuc_1Hex_6HexNAc_5$	C	0.351 ± 0.086	0.241 ± 0.103	0.071 ± 0.044
$Fuc_1Hex_7HexNAc_6$	C	0.398 ± 0.084	0.134 ± 0.082	0.043 ± 0.028
$NeuAc_2Hex_6HexNAc_5$	C	0.248 ± 0.096	0.274 ± 0.050	0.249 ± 0.238
$NeuAc_1Fuc_6Hex_4HexNAc_4$	C	0.025 ± 0.016	0	0
$NeuAc_2Fuc_3Hex_5HexNAc_4$	C	0.011 ± 0.011	0	0
$NeuAc_1Hex_7HexNAc_6$	C	0.091 ± 0.030	0	0
$NeuAc_2Fuc_1Hex_6HexNAc_5$	C	1.228 ± 0.269	1.670 ± 0.932	0.345 ± 0.328
$NeuAc_1NeuGc_1Fuc_1Hex_6HexNAc_5$	C	0.126 ± 0.036	0.210 ± 0.097	0.037 ± 0.057
$NeuAc_1Fuc_1Hex_7HexNAc_6$	C	0.588 ± 0.147	0.321 ± 0.242	0.058 ± 0.049
$NeuAc_1Hex_8HexNAc_6$	C	0.075 ± 0.029	0.042 ± 0.042	0.000 ± 0.013
$NeuGc_2Hex_6HexNAc_6$	C	0.014 ± 0.014	0.058 ± 0.058	0

(Continued)

TABLE 1 | Continued

Glycan composition	Type[a]	Average relative abundance[b]		
		MSC	Osteoblast	21 day basal
$NeuAc_3Hex_6HexNAc_5$	C	0.657 ± 0.268	2.034 ± 1.445	0.027 ± 0.033
$NeuAc_2NeuGc_1Hex_6HexNAc_5$	C	0.045 ± 0.027	0.288 ± 0.194	0
$NeuAc_2Hex_7HexNAc_6$	C	0.077 ± 0.041	0.092 ± 0.092	0
$NeuAc_1NeuGc_1Hex_7HexNAc_6$	C	0	0.054 ± 0.054	0
$NeuAc_3Fuc_1Hex_6HexNAc_5$	C	0.373 ± 0.114	0.993 ± 0.611	0.089 ± 0.135
$NeuAc_2NeuGc_1Fuc_1Hex_6HexNAc_5$	C	0.036 ± 0.015	0.167 ± 0.100	0.014 ± 0.028
$NeuAc_2Fuc_1Hex_7HexNAc_6$	C	0.520 ± 0.176	0.735 ± 0.587	0.068 ± 0.104
$NeuAc_1NeuGc_1Fuc_1Hex_7HexNAc_6$	C	0.038 ± 0.025	0.088 ± 0.076	0.009 ± 0.018
$NeuAc_1Fuc_1Hex_8HexNAc_7$	C	0.047 ± 0.030	0	0
$NeuAc_4Hex_6HexNAc_5$	C	0.083 ± 0.043	0.377 ± 0.255	0
$NeuAc_1Fuc_1Hex_7HexNAc_8$ or[c] $NeuAc_3NeuGc_1Hex_6HexNAc_5$	C	0	0.065 ± 0.040	0
$NeuAc_3Hex_7HexNAc_6$	C	0.023 ± 0.014	0.037 ± 0.037	0
$NeuAc_2Hex_8HexNAc_7$	C	0.004 ± 0.004	0.024 ± 0.024	0
$NeuAc_1NeuGc_1Hex_8HexNAc_7$ or[c] $NeuGc_2Fuc_1Hex_7HexNAc_7$	C	0	0.016 ± 0.016	0
$NeuAc_3Fuc_1Hex_7HexNAc_6$	C	0.174 ± 0.077	0.427 ± 0.332	0.024 ± 0.057
$Fuc_2Hex_8HexNAc_9$ or[c] $NeuAc_3Hex_8HexNAc_6$	C	0	0.057 ± 0.047	0.000 ± 0.009
$NeuAc_2Fuc_1Hex_8HexNAc_7$	C	0.033 ± 0.020	0.070 ± 0.060	0.000 ± 0.006
$NeuAc_4Fuc_1Hex_7HexNAc_6$	C	0.046 ± 0.024	0.233 ± 0.177	0.005 ± 0.017
$NeuAc_3Fuc_1Hex_8HexNAc_7$	C	0.016 ± 0.010	0.061 ± 0.051	0
$NeuAc_2Fuc_1Hex_9HexNAc_8$	C	0.008 ± 0.005	0.013 ± 0.013	0
$NeuAc_4Fuc_1Hex_8HexNAc_7$	C	0.002 ± 0.002	0.019 ± 0.014	0
$NeuAc_3Fuc_1Hex_9HexNAc_8$	C	0.001 ± 0.001	0.011 ± 0.011	0
$Fuc_1Hex_2HexNAc_2$	DP[d]	0.745 ± 0.190	0.746 ± 0.221	1.955 ± 0.311
$Hex_3HexNAc_2$	DP	0.891 ± 0.220	1.053 ± 0.422	2.830 ± 0.603
$Fuc_1Hex_3HexNAc_2$	DP	2.402 ± 0.504	3.291 ± 1.230	6.714 ± 1.163
$Hex_4HexNAc_2$	DP	0.618 ± 0.106	1.216 ± 0.282	1.641 ± 0.323
$Fuc_1Hex_4HexNAc_2$	DP	0.396 ± 0.095	0	0.571 ± 0.227

[a] *Glycans grouped as oligomannose (O), hybrid (H), complex (C), and degradation product (DP).*

[b] *Percentage of total glycan signal ± SEM for n = 5 (MSC), n = 3 (osteoblast) and n = 2 (21 day basal) are shown.*

[c] *Given the mass of these glycans other glycan compositions are possible, but the most plausible two possibilities are shown.*

[d] *DP are small glycans that are not produced by the mammalian N-glycosylation machinery and therefore most likely originate from lysosomal degradation.*

medium (**Figure 3D**, fifth and sixth bars). Thus although Y202 cells do not differentiate into mineral-depositing osteoblasts, they do undergo a significant change, which results in a glycan profile alteration similar to that of osteoblastic Y101 cells.

As a second test we wondered if glycosylation changes are necessary for differentiation into osteoblasts to take place. We used the glycan-processing inhibitor swainsonine, which inhibits mannosidase II. As expected, this caused a major shift in the glycan profile, in which complex glycans almost completely disappeared, and were replaced by hybrid ones (**Supplementary Figure 4**). This allowed us to test whether the shift to complex glycans is required for osteogenesis. While there was a slight reduction in the amount of mineral deposition when the cells where grown in osteogenic medium in the presence of swainsonine, the cells were clearly still capable of forming osteoblasts and depositing calcium phosphate mineral (**Figure 3E**).

DISCUSSION

A previous glycomic study concluded that oligomannose glycans are more abundant in parental stem cells than in their differentiated osteoblasts (Heiskanen et al., 2009). As the glycans in that study were not permethylated a true quantitative assessment of the relative abundance of the various glycan species was not possible (Wada et al., 2007). Our study now provides a detailed quantitative comparison of the MSC and osteoblast glycan profiles of clonal lines made possible by the FANGS method. We find that oligomannose and complex glycans are close to equally abundant in MSCs, while this changes to an almost four-fold higher abundance of the complex glycans in osteoblasts. Shifts in the glycan profile upon differentiation have been reported using quantitative methods such as the MALDI-MS of permethylated glycans and LC-MS of fluorescently labeled glycans (Wada et al., 2007; Hasehira et al., 2012). Comparing

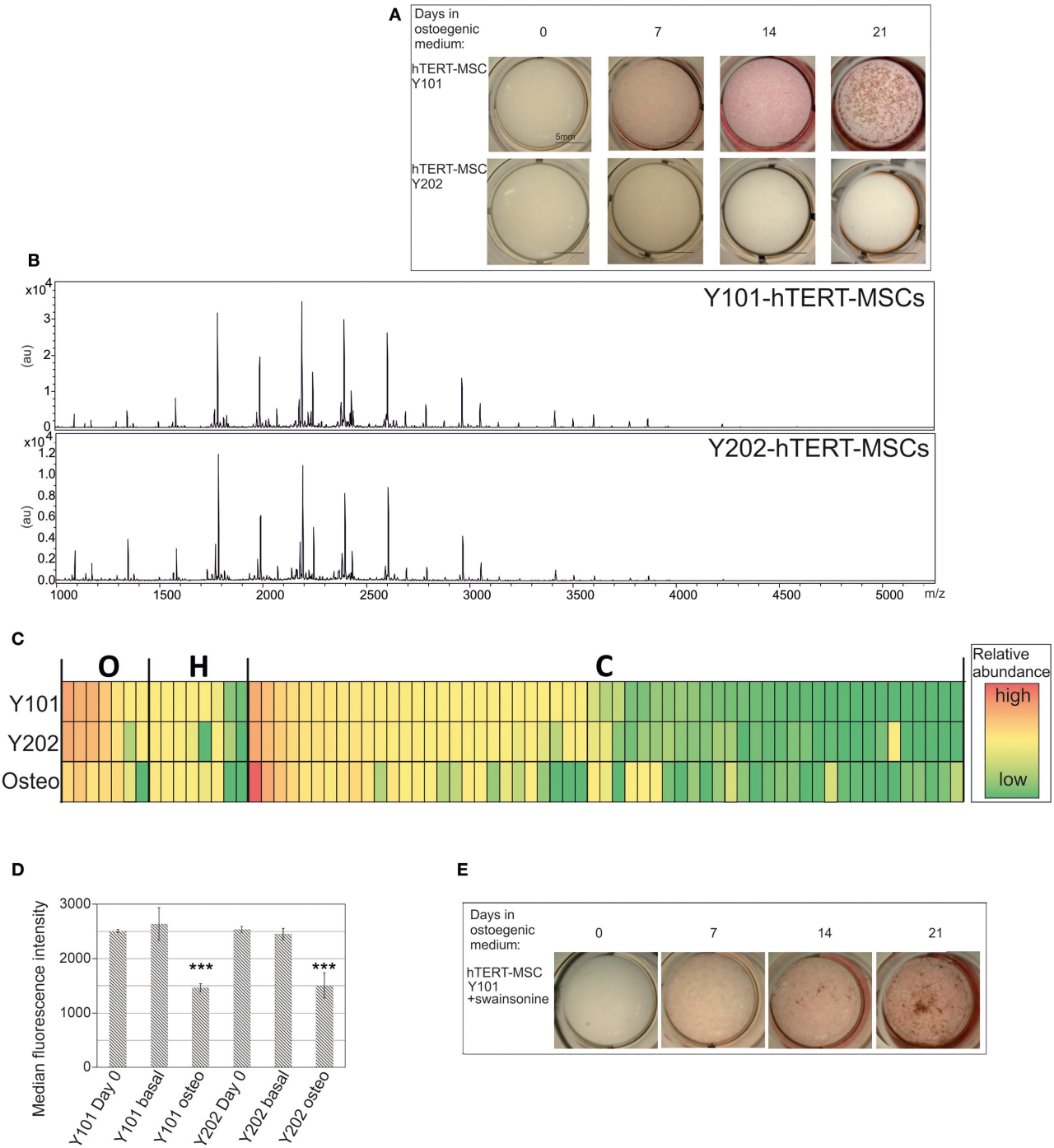

FIGURE 3 | *N*-glycan profile of MSCs does not correlate with differentiation capacity. (A) Osteogenic capacities of Y101 and Y202 hTERT-MSC lines. For details see **Figure 2A**. **(B)** *N*-glycan profiles of the Y101 and Y202 hTERT-MSC lines following 24 h culture in basal medium. Representative MALDI-TOF spectra as in **Figure 1**, but glycan assignments are omitted for clarity. **(C)** Heat map display comparing the *N*-glycan profiles of Y101, Y202 hTERT-MSCs, and osteoblasts derived from the Y101 hTERT-MSCs. Averaged (Y101, Y202 $n = 5$, osteoblasts $n = 3$) normalized peak intensities were compared. Each row in the heat map display represents a single glycan structure. Glycans are grouped by type: oligomannose (O), hybrid (H), or complex (C), and sorted by abundance in the Y101 profile within each type. The cells' colors denote glycan abundance as indicated in the legend. **(D)** Y101 or Y202 cells were grown for 0 or 8 days in basal or osteogenic (osteo) medium, then single cell suspensions stained with ConA-FITC and DAPI and analyzed by flow cytometry. The median intensity of the FITC fluorescence of live cells is shown with SEM from three independent replicates. **(E)** Y101 cells were cultured in basal medium containing 10 μg/mL swainsonine for 48 h before addition of osteogenic medium containing swainonsine at the same concentration. Mineral deposition and ALP activity were tested by ALP/von Kossa staining 0, 7, 14, and 21 days after addition of osteogenic medium. ***$P < 0.001$.

neuronal stem cells and their differentiated progeny, (Yagi et al., 2012) or embryonic stem cells and embryoid bodies (Nairn et al., 2012), showed reductions in oligomannose glycans in the differentiated cells. Along similar lines, the reverse process, generation of induced pluripotent stem (iPS) cells, increased the oligomannose proportion of the glycan profile (Hasehira et al., 2012). None of these comparisons reported a shift in the proportion of oligomannose vs. complex glycans as large as that observed in our study. A likely explanation is that most studies have not compared terminally differentiated specialized cells, but intermediate or mixed stages of differentiation. This possibility is corroborated by the glycomic profiles published for embryonic stem cells and terminally differentiated fibroblasts (An et al., 2012). This latter study used clonal lines, just as our study does, but did not use permethylated glycans, so their quantification is imperfect. Moreover, the fibroblasts investigated were not derived from the stem cells analyzed, so the comparison is largely between a generic stem cell and a generic differentiated cell population, rather than the consequence of the differentiation process *per se* as in our study. Nevertheless, the shift in oligomannose glycan abundance from 70 to 80% in the stem cells to 30% in the fibroblasts (An et al., 2012) is close to the range that we now report. Our results would therefore be in line with the hypothesis that stemness may require oligomannose glycans (Heiskanen et al., 2009; Hamouda et al., 2013).

Another change within the glycan profile is increased overall fucosylation intensity in osteoblasts. Single fucosylation could be present either on the core or the antennae, but glycans with multiple fucoses have to contain fucose on the antennae. From MSCs we observed several glycans with multiple fucoses as opposed to osteoblasts where single fucosylation predominates. Thus while the overall intensity of fucosylation is elevated in osteoblasts, the level of fucosylation on antennae is likely higher in the stem cells, leading us to speculate that fucosylation on the antennae could be a property of stem cells rather than osteoblasts (**Figures 1**, **2B** and **Table 1**). Indeed, iPS cells have been shown to have a lower degree of core fucosylation but more on the antennae than their differentiated parents (Hasehira et al., 2012). In addition, Fut9-dependent fucose addition to the antennae has been implicated in embryonic stem cell self-renewal (Li et al., 2009).

Comparison of the two MSC lines shows a remarkable similarity in their *N*-glycan profiles, despite a marked difference in their differentiation capabilities. The profiles of the stem cell lines we report are also similar to the previously reported profiles of primary MSCs (Heiskanen et al., 2009; Hamouda et al., 2013). We would therefore argue that *N*-glycan profiles are a good indicator of overall MSC identity. Importantly, changes to the glycan profile following prolonged culturing of the stem cells are also small, enforcing the usefulness of glycans as an MSC marker. The main difference between the glycan profiles of MSCs before and after prolonged culturing is a significantly higher abundance of degradation products (**Table 1**). The appearance of such *N*-glycans that cannot be made during biosynthetic processing in mammals is most likely a consequence of intermediates accumulating in the lysosome during glycan degradation (Uematsu et al., 2005). These are picked up in our analysis due to the FANGS process capturing all cellular protein-linked *N*-glycans. The amount of these glycan species in the MSCs and osteoblasts is in line with the levels of paucimannose glycans found in other glycan profiling studies of cultured cells (Abdul Rahman et al., 2014). The increased proportion of this glycan class in the cells that were cultured for 21 days may be explained by increased autophagy and/or cell death, possible consequences of over-confluency due to the lack of differentiation. A further conclusion we can make from these observations is that the differences between MSC and osteoblast glycan profiles are not due to the extended culture time, but rather are genuine differentiation-associated changes.

A major question in glycobiology is the function of glycans during cellular processes. While it is well documented that there are changes in glycan profiles between cell types, the functional consequence of these changes is unclear. Our results suggest that the shift to complex *N*-glycans in MSCs is neither required nor sufficient alone to drive osteogenesis. Inhibition of complex *N*-glycan formation by swainsonine does not inhibit osteogenesis. In contrast, the shift to a lower proportion of oligomannose glycans in Y202 cells is not accompanied by osteoblast mediated mineralization. Rather, it could more likely be the stem-like properties of MSCs that may benefit from certain glycan features.

Our detailed analysis of two MSC cell lines allows us to speculate about the requirements for the various glycan subtypes of different stem cell functions. Given that the Y202 line, which is unable to differentiate effectively, has the same *N*-glycan profile as the differentiating Y101 MSC line, it is unlikely that glycans directly determine the differentiation potential of these cells. However, continued proliferation and self-renewal are also very important properties of stem cells. Indeed terminal fucosylation has been suggested to be an important factor in stem cell maintenance (Li et al., 2009). It is therefore a possibility that some of the features of the profiles found in both the differentiating and undifferentiating MSC lines, which are absent from osteoblasts, such as increased oligomannose content or the fucosylation of antennae, may contribute to the maintenance of a proliferative phenotype.

AUTHOR CONTRIBUTIONS

KW, PG, and DU designed the study; KW performed the experiments; KW, JTO, and DU analyzed the data; KW, JTO, PG, and DU wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcell.2016.00052

Supplementary Table 1 | Comparison of averaged glycan abundances for the hTERT-MSC lines Y101 and Y202.

Supplementary Figure 1 | Representative MALDI-TOF spectrum of permethylated *N*-glycans harvested from MSCs grown for 21 days in basal medium. For details see **Figure 1**.

Supplementary Figure 2 | Comparison of Y101 and Y202 *N*-glycans, for details see Figures 2C–F. Comparisons of averaged (n = 5) Y101 and Y202 normalized *N*-glycan total peak intensities of: **(A)** Individual glycan structures with abundances above 3% of the total. **(B)** Individual glycan structures with abundances of 1–3% of the total. D.P, degradation product, most likely produced in the lysosome. **(C)** Sums of different glycan types. **(F)** Sums of all fucosylated glycan abundances. Error bars show standard error of the mean.

Supplementary Figure 3 | Presence and absence of the indicated cell surface CD markers was determined for Y202 cells using flow cytometry.

Supplementary Figure 4 | Comparison of representative MALDI-TOF spectra of *N*-glycans harvested from Y101 MSCs cultured for 48 h in the presence or absence of swainsonine. The assigned glycan structures are only shown for the most prominent peaks.

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REVIEW
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Stem cell therapy and tissue engineering for correction of congenital heart disease

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This review article reports on the new field of stem cell therapy and tissue engineering and its potential on the management of congenital heart disease. To date, stem cell therapy has mainly focused on treatment of ischemic heart disease and heart failure, with initial indication of safety and mild-to-moderate efficacy. Preclinical studies and initial clinical trials suggest that the approach could be uniquely suited for the correction of congenital defects of the heart. The basic concept is to create living material made by cellularized grafts that, once implanted into the heart, grows and remodels in parallel with the recipient organ. This would make a substantial improvement in current clinical management, which often requires repeated surgical corrections for failure of implanted grafts. Different types of stem cells have been considered and the identification of specific cardiac stem cells within the heterogeneous population of mesenchymal and stromal cells offers opportunities for *de novo* cardiomyogenesis. In addition, endothelial cells and vascular progenitors, including cells with pericyte characteristics, may be necessary to generate efficiently perfused grafts. The implementation of current surgical grafts by stem cell engineering could address the unmet clinical needs of patients with congenital heart defects.

Keywords: congenital heart disease, scaffold, stem cells, tissue engineering, biomaterial

Introduction

Congenital heart disease (CHD) is defined as an abnormality in heart structure that occurs before birth, while the fetus is developing (Sun et al., 2015). It represents the most common congenital anomaly in newborn babies, with a reported prevalence ranging from 6 to 13 per 1000 live births. In the UK alone there are ~4600 babies born with CHD each year (Tennant et al., 2010; Khoshnood et al., 2012). Despite considerable progresses in surgical techniques and medical management of newborns with CHD, there are still considerable mortality and morbidity associated with

Abbreviations: 3D, 3-dimensional; BMCs, bone marrow-derived cells; BMMNCs bone marrow mononuclear cells; BP, bovine pericardium; CHD, congenital heart disease/defect; CPs, cardiac pericytes; CSCs, cardiac stem cells; ECM, extracellular matrix; ECs, endothelial cells; EPCs, endothelial progenitor cells; HF, heart failure; HLHS, hypoplastic left heart syndrome; HPs, heart pericytes; iPS, induced pluripotent stem cells; IVC, inferior vena cava; LV, left ventricle; LVOT, left ventricle outflow tract; MI, myocardial infarction; MMPs, matrix metalloproteinases; MSCs, mesenchymal stem cells; PCL, poly-Caprolactone; PGA, poly-Glicolyc Acid; PLLA, poly-L-Lactic Acid; RA, right atrium; RV, right ventricle; RVEF, right ventricle ejection fraction; RVOT, right ventricle outflow tract; TE, tissue engineering; ToF, Tetralogy of Fallot; UCBMNCs, umbilical cord blood mononuclear cells; VEGF(R), Vascular Endothelial Growth Factor (Receptor); VICs, valve interstitial cells; VSMCs, Vascular Smooth Muscle Cells.

severe forms of CHD, which comprise the first cause of mortality by congenital abnormalities (Khoshnood et al., 2012).

In the last decade, clinical needs of CHD have extended to the adulthood. Recent estimations indicate that 80% of neonates and infants with CHD can expect to reach adulthood (Woodward, 2011; Khoshnood et al., 2012). According to the Department of Health, in 2006 there were around 135,000 adults living in England with CHD (http://webarchive.nationalarchives.gov.uk/). The 32nd Bethesda Conference report estimated that there were approximately 2800 adults with CHD per 1 million population and that more than half of them have a moderate or high complexity defect (Baumgartner et al., 2010). These patients often develop heart dysfunction and failure as well as neurological, respiratory and coagulation problems (*British Heart Foundation Statistics Database: www.heartstats.org*). The economic and social burden of CHD is high and rapidly increasing. In 2004, the U.S. hospital costs for CHD totaled $1.4 billion (Henaine et al., 2012).

Typical congenital abnormalities comprise valves defects, atrial and ventricular septa defects, stenosis and alterations of the aorta and pulmonary veins and arteries and heart muscle abnormalities. The defects can range in severity from relatively simple problems, such as holes between chambers of the heart that can be surgically closed, to very severe malformations, such as the complete absence of one or more chambers or valves, which cause deficits in blood oxygenation and circulation, heart failure (HF), and eventually premature death (Woodward, 2011; Sun et al., 2015). Common *single CHD* are represented by holes (intra-cardiac shunts) inside the internal wall of the heart (**Figure 1**, left): in *septal defects*, oxygenated blood returning from the pulmonary veins flows from the left to the right chambers of the heart, where it mixes with deoxygenated blood returning from the body, finally causing an overloading of the right ventricle (RV) (Geva et al., 2014; Sun et al., 2015). With time this overload induces remodeling of pulmonary vasculature and hypertension with consequent inversion of the shunt and cyanosis (Woodward, 2011; Sun et al., 2015). When mixed lesions are present at the same time the pathology increases in severity and results in *complex CHD* (**Figure 1**, right). The most common one is *Tetralogy of Fallot (ToF)*, which features four cardiac abnormalities: narrowing of the pulmonary outflow tract, a hole connecting the ventricular chambers, RV hypertrophy, and the aorta that lies over the hole between ventricular chambers. In ToF patients, an outflow obstruction prevents the blood flowing from the RV into the pulmonary artery, causing deoxygenated blood to pass through the ventricular hole into the left ventricle (LV) and then into the aorta (Woodward, 2011; Wald et al., 2014). Another very serious condition is the *hypoplastic left heart syndrome (HLHS)* (or univentricular heart syndrome), characterized by hypoplasia of the LV, the aorta and related valvular components, with systemic flow becoming dependent on a patent ductus arteriosus. In this condition, blood returning to the heart from both the systemic circulation and the lungs mixes before being pumped by the RV to both the systemic and pulmonary circulation, causing severe cyanosis, increased pressure workload and ultimately failure of the RV (Barron et al., 2009).

This review focuses on stem cell therapy and tissue engineering as a new option to implement current surgical methods for definitive correction of CHD. The approach was initially conceived with the objective to repair and/or replace damaged tissues and organs. However, stem cells from young individuals possess superior naivety and plasticity than adult stem cells and could be better suited for regenerative purposes. The use of scaffolds engineered with stem cells may offer unprecedented therapeutic opportunities for addressing unmet clinical needs of patients with complex cardiac defects.

Elective Surgical Correction

The ideal therapeutic option for CHD patients is one-step corrective surgery, during which the heart surgeon closes holes in the heart with stitches or a patch, repairs or replaces valves, widens arteries, and restores the proper location of major blood vessels (Sun et al., 2015). In patients with ToF, the definitive goals are relief of all obstruction to blood flow from the RV to the pulmonary artery and closure of the ventricular septum defect. Reconstruction of RV outflow tract (RVOT) obstruction may involve resection of obstructing muscle bundles, creation of an RVOT patch, pulmonary valvotomy or valvectomy, and pulmonary arterioplasty (Henaine et al., 2012). However, complex CHD usually require more than one open-heart surgery to correct the structural alterations (Woodward, 2011). On the one hand, palliative procedures may be indicated to relieve symptoms of acute HF, allowing definitive correction to be performed when the baby has gained weight and hemodynamics are stabilized (Yuan and Jing, 2009). For instance, babies with HLHS require a surgical palliation within few days from birth as the risk of death is 95% within few weeks from birth without any treatment (Barron et al., 2009; Frescura and Thiene, 2014; Ishigami et al., 2015). On the other hand, multiple re-interventions become often necessary because of deterioration of the implanted grafts (Said and Burkhart, 2014). Patients at the highest risk of death and not suitable for reparative or palliative surgery are candidate to heart transplantation, this extreme option being limited by shortage of donors (Razzouk and Bailey, 2014; Hsu and Lamour, 2015; Ishigami et al., 2015; Sun et al., 2015).

Limitations of Current Surgical Approach

The use of prosthetic materials in the form of conduits, patches and new valves made by xenografts, homografts, or autografts is routine in congenital cardiac surgery. Even though these grafts may be life-saving, they are characterized by some limitations, represented by a limited durability, and the risks of infection, host immune response, and thrombotic complications. A crucial problem still to be overcome in the pediatric population is the lack of growth and remodeling potential of the grafts currently used for CHD surgery (Mirensky and Breuer, 2008). In the following paragraphs, we illustrate the advantages and disadvantages of clinically available grafts. Additionally, these aspects will be reconsidered in the perspective of creating cellularized scaffolds in a subsequent section of this review.

Xenografts are biological grafts deriving from animals, commonly porcine and bovine, largely used in surgery because

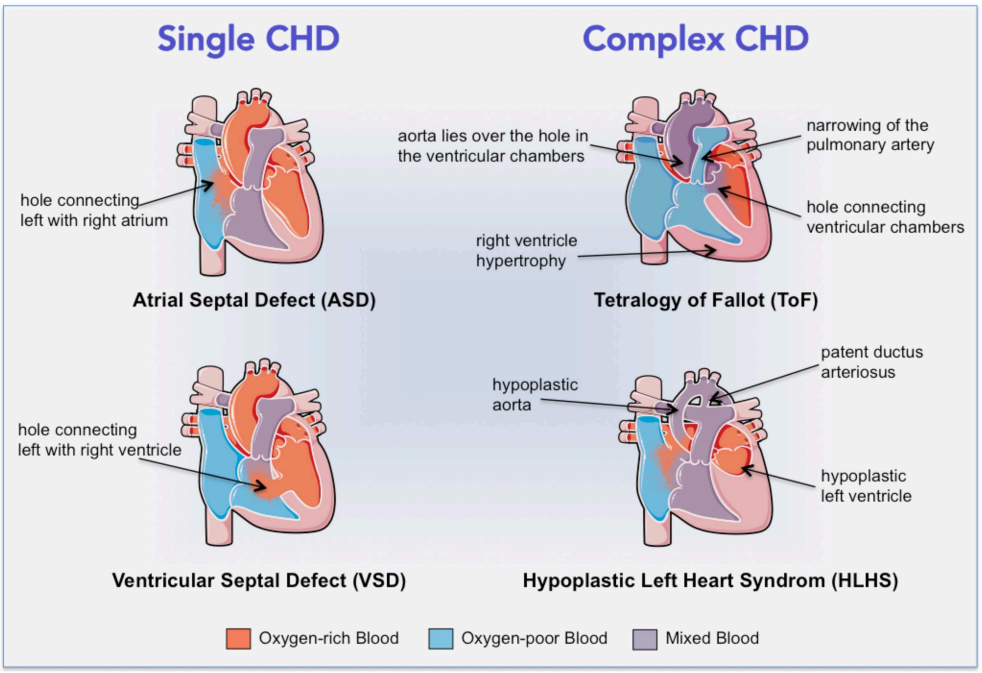

FIGURE 1 | Cartoon illustrating the cardiac structural alterations in common single and complex CHD.

of the shortage of human substitutes. Xenogenic bovine pericardium and porcine valves remain the first choice for heart valve substitution (Yap et al., 2013). The porcine valve has the advantage of adequate anatomic structure and unlimited availability. The bovine pericardium contains a higher amount of layered structural proteins than autologous human pericardium, giving it elastic properties that allow conformity to complex anatomical geometries, optimal for RVOT reconstruction (Pok and Jacot, 2011; Strange et al., 2015). In order to avoid the activation of the recipient's immune response, xenografts are decellularized, a process during which animal cells are removed from the graft while the extracellular matrix is preserved, to provide the remaining scaffold with the original anatomical structure (e.g., valve) and the adequate support for the recolonization by the patient's cells after implantation. Different methods have been used for this purpose, among which there are enzymatic cell lysis (Trypsin), detergents and chemicals cell removal (Sodium dodecyl sulfate, Sodium Azide, and Sodium deoxycholate), freeze drying, and a combination of chemical and enzymatic methods (Rieder et al., 2004; Schmidt et al., 2007; Tudorache et al., 2007). An additional type of xenograft manufacture includes cross-linking with chemicals (such as glutaraldehyde), a process by which proteins are cross-linked and collagen fibers stabilized, conferring the graft with tensile strength, elasticity and resistance to degeneration; due to their cytotoxicity, these chemicals eliminate also xenogenic cells (Schmidt et al., 2007; Butcher et al., 2011). However, improvements in pliability and tolerogenicity come at a price (**Figure 2**). In fact, elimination of valve interstitial cells (VICs), which normally ensure the regular turnover of the valve extracellular matrix (ECM), makes prostheses more susceptible to degeneration, both *in vitro* and after implantation in a mechanical environment. The seeding of VICs onto the decellularized scaffolds has been proposed to overcome this problem; furthermore, the reseeding of the graft using cells able to produce new ECM is crucial to prevent the degeneration of the graft structure (Cushing et al., 2005). Noteworthy, while decellularized grafts are metabolized and remodeled by matrix-metalloproteinases (MMPs) after implantation in the patient, cross-linked grafts do not allow MMPs degradation, thus interfering with the remodeling process (Schmidt et al., 2007). In fact, to preserve the mechanical properties of biological grafts, the remodeling process should be balanced between the matrix formation and degradation. Last, atherosclerotic processes also participate in prosthetic valve remodeling, with initial accumulation of oxidized low-density lipoproteins, followed by monocyte recruitment, generation of a pro-inflammatory milieu, collagen disruption and osteogenic differentiation of resident ECs and precursor cells recruited from the circulation (Shetty et al., 2009; Gossl et al., 2012).

Cryopreserved homografts—usually valves or vascular conduits—are derived from humans, commonly cadavers. They are the heart valve replacements closest to the natural valve, being non-thrombogenic and having a low risk of infection. Cryopreserved homografts have been used for many years in CHD surgery, for pulmonary or aortic valve replacement, or for RVOT reconstruction during the Ross operation (Gabbieri et al., 2007; Neumann et al., 2013). Homografts are not chemically cross-linked and exhibit good mechanical properties. Disadvantages are their limited availability, more difficult

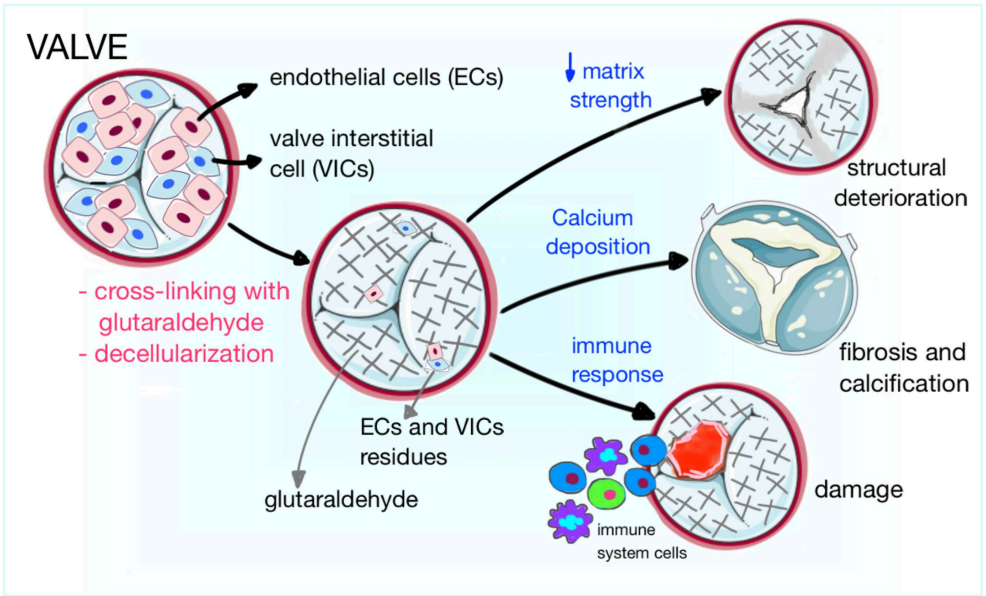

FIGURE 2 | Cartoon illustrating the mechanisms of prosthetic valve degeneration. Xenogenic or allogenic valves are decellularized to reduce the risk of immune response and rejection. In addition, animal derived valves can be cross-linked with glutaraldehyde. The three main mechanisms of valve failure are structural deterioration, fibrosis and calcification, and damage by rejection from the host immune system.

implantation techniques and failure associated with a specific immune response, especially in young individuals. Although homografts have been associated with a very good hemodynamic performance, the cryopreservation and the thawing process can produce the structural deterioration of the matrices, limiting the durability of the graft (Neumann et al., 2013). In the younger patients, the risk of structural valve deterioration ranges from 71 to 87% at 10 years (Ruel et al., 2005).

Decellularized homografts have been proposed as a good alternative, showing lower explantation and degeneration rates than conventional cryopreserved valve grafts (0–10% vs. 30% after 5 years, respectively) (Cebotari et al., 2011; Ruzmetov et al., 2012). Additionally, implantation of decellularized homografts is reportedly associated with spontaneous recellularization (da Costa et al., 2005; Dohmen et al., 2006b; Cebotari et al., 2011).

Autografts are created using the patient's own tissues. Pulmonary autografts are commonly used in children and young adults with ToF or valves defects. During the Ross procedure, the surgeon replaces the aortic valve with the autologous pulmonary valve of the patient, and performs the reconstruction of the RVOT using a prosthetic valve (Al-Halees et al., 2002). A study in 30 children demonstrated the growth of the pulmonary autograft in parallel with somatic growth (Simon et al., 2001). However, autografts are prone to dilatation with subsequent aortic valve regurgitation (Dohmen et al., 2002). The freedom from RVOT replacement for children is reported to be about 90% at 12 years (Pasquali et al., 2007). Independently of the material used, *prosthetic reconstruction of RVOT* is often associated with mechanical and electrical abnormalities arising from non-contractile/non-conductive patch material (Tweddell et al., 2000; Perri et al., 2012).

Autologous pericardium is easily harvested during cardiac surgery, but is difficult to handle since it has a propensity to roll at the edges rendering the procedure difficult and time-consuming (Pok and Jacot, 2011).

Synthetic Gore-Tex and *mechanical valves* are durable but lack of growth potential; moreover, mechanical valves require chronic anticoagulation treatment to reduce the risk of thrombosis. Careful monitoring of coagulation parameters is necessary to avoid bleeding complications (Said and Burkhart, 2014).

Considering limitations and limited durability of available prostheses, there is an urgent need of new therapeutic strategies to optimize long-term outcomes in CHD patients. Recent advances in regenerative medicine allow the manipulation of a spectrum of stem/progenitor cells, including endothelial, mesenchymal, and cardiac stem cells, to support the youngest heart repair. In the following sections, we will report two main approaches of regenerative medicine, namely stem cell therapy (transplantation of dispersed stem cells) and tissue engineering (use of grafts functionalized with single or multiple stem cell types). Stem cell therapy has provided first proof-of-concept, thus paving the way to tissue engineering to fabricate cellularized valves endowed with anti-coagulative properties and living cardiac tissue that grows physiologically in parallel with the heart's growth.

Stem Cell Therapy for CHD

Cell therapy holds promises for treatment of a range of disabling diseases. This therapeutic strategy is based on the injection of dispersed cells in the site of damage, aimed at stimulating the regeneration of the damaged tissue and possibly the recovery

of its function. In the heart, different ways of delivery can be used, that are intracoronary, intramyocardial, intravenous, and epicardial. The therapeutic effects can be determined by a direct cellular effect, given by transdifferentiation of injected cells in cardiovascular cells, or more likely by a cytokine-paracrine effect, that means cells release soluble factors that exert protective effects on neighboring cells, although not participating in the formation of new functional tissue by trans-differentiation (Krause et al., 2010). From recent studies a new paracrine mechanism has emerged, based on the release of intracellular material—mainly proteins and micro-RNA—by particular vescicles called exosomes (Ibrahim et al., 2014).

In the cardiovascular medicine field, stem cell-based therapies have been principally applied to the treatment of adult patients with myocardial ischemia or HF (reviewed in Sanganalmath and Bolli, 2013). The first two randomized phase I clinical trials, SCIPIO and CADUCEUS, in which respectively c-Kit-positive Cardiac Stem Cells and Cardiosphere-derived Cells were administered to end-stage HF adult patients, showed positive outcomes regarding safety and efficacy of cell therapy (Bolli et al., 2011; Makkar et al., 2012). Only recently, the approach has been proposed for correction of congenital cardiac defects (Bernstein and Srivastava, 2012; Pincott and Burch, 2012).

Which Cell Type Should Be Used?

A comprehensive solution for complex cardiac defects like ToF requires the generation of valves and grafts containing a spectrum of regenerative cells. On the one hand, the use of multipotent stem cells able to generate all the components of the cardiac tissue, i.e., vascular cells, cardiomyocytes, and stromal cells, represents an option. On the other hand, the combination of different progenitor cells with restricted and predictable lineage commitment might be preferred by regulatory agencies for safety reasons. Hence, at this stage, research on different cell types should be pursued.

Induced Pluripotent Stem Cells

Induced pluripotent stem cells (iPS) are pluripotent cell lines obtained from the *ex vivo* reprogramming of fetal or adult somatic cells, like fibroblasts. During the reprogramming process, commonly four classical recognized pluripotency factors (NANOG, OCT-4, c-MYC, and KFL-4) are introduced within the cells from an ectopic source, usually carried by retroviral vectors. Reprogrammed cells are characterized by a high plasticity, being able to differentiate in cells of the three embryonal germ layers and thus potentially having the capacity to differentiate in most of the human body's cells (Takahashi et al., 2007; Yu et al., 2007). These last properties make iPS a good choice for application in regenerative medicine. For employment in CHD surgery, iPS derived from the patient's cells could be ideally differentiated *in vitro* in vascular and myocardial cells before autologous transplantation (Lundy et al., 2013; Yoder, 2015). To date, the limitation to the clinical use of these cells is given by the risk of tumorigenesis deriving from the genomic integration of the viral vectors (Mayshar et al., 2010).

Foetal and Umbilical Cord Cells

The time of diagnosis is key for the decision. Owing to advances and diffusion of pre-natal cardiac imaging, it is now possible to recognize cardiac defects in a large proportion of subjects. For these cases, foetal cells and umbilical cord cells represent valid therapeutic candidates and, in the future, *in utero* repair of cardiac defects using these cells will become a routine practice. Umbilical cord is collected at the time of birth; umbilical cord blood mononuclear cells (UCBMNCs) can be isolated from the blood, while mesenchymal stem cells are extracted from the Wharton's jelly. Cord stem cells are able to differentiate in cardiomyocyte-like cells and endothelial cells (ECs) (Chen et al., 2009; Wu et al., 2009). Last year, the Mayo Clinic announced the first U.S. stem cell trial with autologous umbilical cord blood cells to treat children with HLHS (http://www.mayo.edu). Foetal-derived stem cells can be isolated from the amniotic fluid and include both pluripotent stem cells and more committed cells (Klemmt et al., 2011). Foetal cells could be stored, according to the large experience accumulated with umbilical cord blood cells, and used for multi-stage corrections.

Adult Cells

Despite the promising potential of iPS and foetal cells, so far most preclinical and clinical studies have employed post-natal cells for both safety reasons and easy accessibility to adult tissues. Different cell types derived from post-natal tissues can be used.

Endothelial cells and progenitor cells

Initial focus of research has been the re-endothelialization of cardiac valves to reduce thrombotic complications. Optimal candidates for this purpose are ECs and endothelial progenitor cells (EPCs). The first can be isolated from peripheral, easy accessible veins, such as the saphenous or forearm veins, while the second ones can be purified from the peripheral blood or bone marrow. EPCs can circulate in peripheral blood and can be incorporated in regions of active neovascularization, such as the ischemic myocardium (Xin et al., 2008). Experimental evidence suggests that EPCs participate not only in the process of vasculogenesis substituting the lost ECs but also in the endothelialization of grafts (Young et al., 2007). The significance of EPCs in cardiovascular disease has been reviewed in Madonna and De Caterina (2015).

Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are a heterogeneous subset of stromal stem cells that can be isolated from many adult tissues, including the heart, skeletal muscle, bone marrow and adipose tissue (Uccelli et al., 2008). MSCs stand out as an encouraging option for cell therapy due to their accessible isolation, great expansion potential, immunoregulatory activity and angiogenic properties (Dimarino et al., 2013). Not less relevant, MSCs possess a multipotential differentiation capacity, being able to differentiate, *in vitro*, into cells of the mesodermal lineage (Dominici et al., 2006), and possibly toward cells of endoderm and ectoderm derivation (Uccelli et al., 2008). *In vivo*, although being able to generate new cardiomyocytes and vascular cells, MSCs stimulate vascular and cardiomyocyte regeneration acting

prevalently by paracrine mechanisms (Gnecchi et al., 2006, 2012). Also, they may positively influence cardiac metabolism and contractility (Gnecchi et al., 2012). The immune compatibility of the MSCs is a remarkable advantage for the translation of their use in clinics (Castro-Manrreza and Montesinos, 2015).

Bone marrow-derived progenitor cells

Total bone marrow-derived cells (BMCs) and especially subpopulations of bone marrow mononuclear cells (BMMNCs, that means either MSCs, or hematopoietic stem cells (HSCs), or monocytes) have been used in transplantation trials in acute myocardial infarction (MI) patients (reviewed in Simari et al., 2014). The advantage of using these cells is the easy accessibility with low risks for the patients, and the possibility to harvest high numbers of cells without requirement of long time *in vitro* expansion. The mechanisms by which BMCs can contribute to an improvement of cardiac function after transplantation in the patient heart are still debated, but two main mechanisms have been proposed: (1) paracrine influence on surrounding cardiac cells (Uemura et al., 2006), and (2) variable levels of transdifferentiation in ECs, pericytes, VSMCs and cardiomyocytes (Hosoda et al., 2010; Yoon et al., 2010). Noteworthy, the potential of transdifferentiation in cardiomyocytes still represents an open challenge for stem cell scientists; regarding the BM-MSCs subpopulation, there are discrepancies between scientists who reported the cells ability to generate new cardiomyocytes *in vivo* post-MI (Kawada et al., 2004; Yoon et al., 2005) and those who showed, instead, that BM-MSCs have very limited ability to generate functional contractile cardiac cells (Rose et al., 2008; Beitnes et al., 2009; Dixon et al., 2009; Tendera et al., 2009; Wohrle et al., 2010). Conversely, this cardiogenic ability has been negated to HSCs (Balsam et al., 2004; Murry et al., 2004).

Cardiac stem cells

Harvesting MSCs from accessible sources, like the subcutaneous adipose tissue, represents an attractive option. However, MSCs present in the stroma of different tissues are heterogeneous and may be influenced by the specific environment where they reside. The idea of using more specialized cell populations was pursued with the use of MSCs obtained from the target organ: the heart. Several investigations over the last 15 years have demonstrated that new cardiovascular cells are generated starting from primitive undifferentiated cells (*cardiac stem cells—CSCs*) resident in the heart since from birth, with this phenomenon playing a primary role after an acute injury as a myocardial infarction (Torella et al., 2006; Bearzi et al., 2007; Hsieh et al., 2007; Bergmann et al., 2009). Several CSC classes have been identified in the adult human heart, based on the expression of different markers (Torella et al., 2007; Beltrami et al., 2011; Bernstein and Srivastava, 2012). The most extensively characterized CSCs express the Stem Cell Factor-receptor (SCF-R, also named c-Kit), the Stem Cell Antigen-1-like (Sca-1) and the Multidrug Resistance-1 (MDR-1, receptor which belongs to the class of ABC transporters that mediate the Hoechst dye efflux from the cell) (Muller et al., 2002; Quaini et al., 2002; Messina et al., 2004; Bearzi et al., 2007). All the above cells do not express transcription factors or membrane and cytoplasmic proteins shared by mature cell types, and thus they are defined as Lineage-negative (Lin-); moreover CSCs are characterized by the capacity of self-renewal, clonogenicity, and multipotency, being able of differentiation in all the mature cardiovascular cell types. All the mentioned properties are essential for the formation of new cardiac tissue, although this latter phenomenon is not sufficient to restore a massive loss of tissue (Muller et al., 2002; Quaini et al., 2002; Messina et al., 2004; Bearzi et al., 2007).

Combinatory cell therapy

The heart is made by different types of cells. Therefore, optimum cell therapy for cardiac repair may require the association of different cell populations with complementary activities leading to balanced cardiomyogenesis/vasculogenesis. Apart from a recent study reporting the benefit of associative treatment with human MSCs and human c-Kit+ CSCs in an immunosuppressed swine model of myocardial infarction (Williams et al., 2013), combinatory cell therapy has received very little attention. We recently reported the advantage of dual therapy with CSCs and vascular pericytes for harmonized repair of the infarcted heart in mice (Avolio et al., 2015a). Interestingly, the two cell populations exert reciprocally enhancing paracrine activities, which lead to increased proliferation of vascular cells and cardiomyocytes and attraction of endogenous CSCs. These seminal examples of combinatory cell therapy set the basis for fabrication of scaffolds functionalized with multiple cell types.

Stem cells in the youngest heart

Despite minor attention has been focused on the pediatric heart, a few studies displayed that CSCs are more abundant in the neonatal period and rapidly decrease over time, making myocardial samples from the youngest hearts an optimal source of stem cells for use in reconstructive surgery (Peral et al., 2014). In particular, the histological examination of RV biopsies from CHD patients aged 2–93 days demonstrated that c-Kit-positive CSCs are four-fold more abundant during the first post-natal month (0.4 vs. 0.1% of total cardiac cells at birth and after 1 month, respectively), and a similar reduction was observed also for NKX2.5-positive cells (Amir et al., 2008). Moreover, Mishra et al., compared c-Kit-positive CSCs isolated from right atrial (RA) specimens of CHD patients selected on the basis of 3 different groups of age: neonates (<30 days), infants (1 month to 2 years) and children (2–13 years), and showed that c-Kit-positive CSCs are two- and three-fold higher in neonates than in infants and children respectively, along with a superior differentiation potential (Mishra et al., 2011). Similarly, a comparison of human hearts aged between 9 days to 77 years showed that the number of CSCs isolated per mg of cardiac tissue was more than three-fold higher in the youngest hearts (Tateishi et al., 2007). In addition to these considerations, a study performed in children aged 19 days to 16 years affected by univentricular heart and chronic pressure overload, showed that the number of c-Kit-positive cells in RV of patients is three-fold higher than in cardiac biopsies post-transplantation, suggesting that the pressure overload may lead to an increase in resident CSCs (Rupp et al., 2012a). This latter may represent an adaptive response in CHD children in the attempt

to counteract the alterations in cardiac function, although not sufficient to overcome the challenges of severe CHD.

But stem cells resident in neonatal hearts are not only more abundant than the adult counterparts. A study performed by Simpson et al., showed that c-Kit/CD90-positive CSCs generated from neonatal RA samples are also endowed with a superior regenerative ability than CSCs derived from adult samples, when transplanted in a rat model of acute myocardial infarction (Simpson et al., 2012). Authors showed that neonatal CSCs are characterized by higher levels of c-Kit, flk-1 and Islet-1-positive cells in comparison with the adult ones; the younger cells possessed also an increased cardiomyogenic potential both *in vitro* and *in vivo*, preventing the formation of the fibrotic scar and improving cardiac functional recovery when transplanted in the animal hearts. Authors supposed that a possible explanation for this augmented regenerative ability of neonatal CSCs might derive from the higher secretion of pro-angiogenic factors, like Vascular Endothelial Growth Factor (VEGF) and Angiogenin, which play a crucial role during the formation of new blood vessels (Simpson et al., 2012).

Besides the above mentioned CSC classes, other cell types have been isolated from the youngest human heart, and may represent a valid option in cell therapy for correction of CHD. Very recently, a population of heart pericytes (HPs) has been identified by the group of B. Péault in myocardial samples from both fetuses at 17–23 weeks of development and post-mortem adults. HPs, recognized as CD34/45-negative and NG2/PDGFR-β/CD146-positive cells, possess a multilineage mesodermal differentiation potential, including limited cardiomyogenic properties both *in vitro* and *in vivo* in a mouse model of MI, and marked pro-angiogenic ability *in vitro* (Chen et al., 2015). Despite this, the potential of these cells has not been evaluated immediately after birth yet. Instead, our group has recently been involved in the isolation and characterization of a population of cardiac pericytes (CPs) from myocardial leftovers of neonates and children operated for correction of CHD (Avolio et al., 2015b). We originally select CPs as CD34-positive CD31-negative cells, and after expansion CPs express pericyte (NG2/PDGFR-β) along with stemness markers (NANOG, OCT-4, SOX-2), are clonogenic and endowed with ability to differentiate into VSMCs, but not into ECs or cardiomyocytes. We demonstrated that CPs are able to attract endothelial and cardiac stem cells in an *in vitro* migration assay, and support the network forming ability of ECs in an *in vitro* angiogenic assay (Avolio et al., 2015b). Last, in the human fetal aorta a population of CD133/CD34/VEGFR-positive vascular progenitor cells has been identified and characterized for its unique vasculogenic and myogenic potential in a mouse model of limb muscle ischemia (Invernici et al., 2007).

Preclinical Trials

Since the complex nature of congenital heart defects makes these latter difficult to reproduce in animals, there are no many animal models of CHD, and this represents a significant limit in the translation of new therapies to clinic (Peral et al., 2014; Tarui et al., 2014). Nevertheless, in the last years some efforts and progresses have been done, in particular to reproduce an increased pressure or volume overload of the RV that is a common feature in patients with a HLHS or ToF. Although it is difficult to make an animal model that mimics univentricular hearts, a pressure-overload right-heart model using pulmonary artery banding has been developed in rats (Hoashi et al., 2009) and sheep (Davies et al., 2010), which is useful to evaluate the safety and efficacy of cell transplantation in presence of single ventricular lesions. In these studies, skeletal myoblasts (Hoashi et al., 2009) and cord blood stem cells (Davies et al., 2010) were injected in the animal hearts, determining an improvement of RV function. Also, a first murine model of RV volume overload has been obtained by Reddy et al., by entrapping the pulmonary valve leaflets with sutures (Reddy et al., 2013). In addition, a recent study performed in a piglet ToF model showed the safety and feasibility of intramyocardial administration of human MesP1 (mesodermal posterior 1)-positive /SSEA-1(stage specific embryonic antigen-1)-positive embryonic stem cell-derived cardiac progenitors 4 months after a surgical procedure mimicking the repair of ToF. This latter consisted of an enlargement of the RVOT by a polytetrafluoroethylene patch, the excision of one pulmonary valve leaflet and a pulmonary artery banding. At 3 months follow-up, animals receiving cell therapy showed some improvements in RV remodeling (reduction of peri-myocyte fibrosis) but unfortunately no significant improvement in RV function compared to control animals (Lambert et al., 2015). Finally, the feasibility and long term safety of autologous UCBMNCs transplanted intramyocardially into the RV of piglet hearts was recently demonstrated (Cantero Peral et al., 2015).

Clinical Trials in Children with CHD

So far, major attention has been dedicated to the therapy of pediatric dilated cardiomyopathy (Rupp et al., 2009; Bergmane et al., 2013; Selem et al., 2013), while experience with stem cell therapy in children with severe CHD or acquired heart failure has been limited to single cases and small-size cohorts.

In 2010, Rupp et al., reported a case of cell therapy with intracoronary injection of autologous BMCs in an 11-months-old boy with HLHS; although this was only an isolated case, the patient outcome was dramatically improved 3 months after cell therapy, with RV ejection fraction (RVEF) increasing from 22 to 44% (Rupp et al., 2010). More recently, intraoperative administration of autologous UCB-stem cells in a 4-months-old baby undergoing a second palliation surgery gave positive results, improving the RVEF from 30 to 50% at 3 months of follow-up (Burkhart et al., 2015). Again, another study performed in 9 children with severe terminal HF (6 with HF secondary to dilated cardiomyopathy and 3 with CHD) showed the feasibility and safety of intracoronary infusion of autologous BMMNCs, stabilizing patients conditions during the short (3 months) and long term (up to 52 months) follow-up (Rupp et al., 2012b).

The first long term follow-up phase I controlled clinical trial in pediatric patients with CHD, using autologous CSCs, has been concluded only recently (Ishigami et al., 2015). In the TICAP trial (Transcoronary infusion of Cardiac progenitor Cells in patients with single ventricle physiology), autologous CSCs were isolated from 7 children affected by HLHS, aged 5 months to 3 years, and administered via intracoronary delivery 4–5 weeks after surgical

palliation. Seven children were selected as control (received palliation surgery without cell therapy). The administration procedure was safe, without any serious adverse event. At 18 months of follow-up, the CSC-treated patients demonstrated an improvement in RVEF from an average baseline value of 47 to 54%, whereas control patients showed little improvement in RVEF, from 47 to 49% (Ishigami et al., 2015). This study gave the first important demonstration of safety and feasibility of autologous CSCs application in young CHD patients. The same group is now carrying out a larger phase II study (Cardiac progenitor Cell infusion to treat univentricular heart disease: PERSEUS), which involves 34 patients randomly assigned to the treatment or control group, to strengthen the positive results collected during the first phase (Tarui et al., 2014).

Tissue Engineering for CHD correction

Tissue engineering is a promising bioengineering technology that aims at the creation of unique substitutes using three-dimensional synthetic or biologic scaffolds seeded with autologous stem cells and differentiated cells, to allow individual patients therapy with regeneration, remodeling and growth potential (Cheema et al., 2012; Smit and Dohmen, 2015). On the one hand, scaffolds serve as a site of cell attachment and new tissue formation. On the other hand, their recolonization by cells after implantation seemingly contributes to the creation of a living tissue perfectly integrated in the recipient's organ. Seeded cells may contribute to the homing of resident cells by secretion of chemoattractant factors.

Tissue engineering has been one of the most promising strategies for the regeneration of impaired tissues. For instance, a tissue engineering approach has been used successfully for the repair of injuries to, or congenital absence of complex organs such as the trachea, esophagus, or skeletal muscle (Macchiarini et al., 2008; Badylak et al., 2012). The first success, in 1994, was the reproduction of tracheal cartilage using tissue engineering techniques (Vacanti et al., 1994). Later, bioengineering techniques have been applied also to the heart. Experience accumulated in adult patients suggests that dispersed stem cells may die soon after implantation in the heart, their therapeutic action being attributable to paracrine factors released during the initial post-transplantation phase. Therefore, this approach does not seem to be advantageous for the repair of complex congenital defects, that frequently requires additional tissue in various forms, such as patches, valves and conduits (Dean et al., 2012; Kalfa and Bacha, 2013; Smit and Dohmen, 2015). Stem cells may work more efficiently if embedded in extracellular matrices, prosthetic grafts and patches to create biological structures that, once implanted in the defective heart, can grow and remodel in a physiologic manner in parallel with cardiac and whole body growth (**Figure 3**) (Bertipaglia et al., 2003; Scholl et al., 2010).

Which Characteristics Should an Optimal Bioengineered Graft Have?

The creation of an optimal bioengineered graft relies upon the careful choice of two main protagonists: (1) the biomaterial and (2) the cells that will repopulate the biomaterial graft. Scaffolds used in cardiac surgery should provide structural integrity at the time of repair, while permitting infiltration of cells from native tissue and enabling the development of new vessels and blood supply, which contributes to tissue remodeling. The cell-scaffold constructs are configured to serve as a template to promote development of structures that closely mimic the original tissues (Cheema et al., 2012; Fallahiarezoudar et al., 2015). Interestingly, physiological cyclic strain and shear stress may even accelerate the formation of tissue starting from the cellularized graft (Engelmayr et al., 2006). The final result should be a new tissue composed entirely of the patient's autologous cells, able to remodel in response to physiological changes (Butcher et al., 2011; Dean et al., 2012). Not less important, an advantage of coating the prosthetic materials with stem cells or ECs before transplantation is the prevention of thrombotic complications.

The optimal reparative patch for closure of atrial and ventricular septal defects and RVOT reconstruction, as well as the optimal substitutive valves used in pediatric CHD, ideally should possess the following characteristics (Butcher et al., 2011; Dean et al., 2012; Kalfa and Bacha, 2013; Alsoufi, 2014):

- absence of immunogenicity,
- growth potential proportional to somatic enlargement,
- excellent durable hemodynamic profile,
- availability in different sizes,
- pliability,
- association with minimal thromboembolism risk thus not requiring anticoagulation,
- low incidence of structural degeneration,
- resistance to calcification.

These characteristics are critical especially in newborns, in which corrections should be durable for all the course of a normal lifespan, to avoid repeated risky operations. But to date, the optimal scaffold still does not exist, although many progresses have been done in this direction, and a wide series of biological and synthetic materials are currently under evaluation in the attempt to find the optimized one.

An important factor to consider during the creation of a bioengineered graft is its mechanical behavior. Before proceeding with any preclinical validation, the performance of bioengineered grafts is first evaluated *ex vivo*. This is done both before and after the seeding of cells, to understand how the cells affect the matrix remodeling in biological grafts. During these tests, the hydrodynamics functions and the durability of the graft are evaluated, both under static conditions and in presence of pulsatile flow. The elasticity and stiffness of the graft are evaluated with both uniaxial and biaxial mechanical tests (Ghanbari et al., 2009).

Adaptability of Currently Available Grafts to Tissue Engineering

Some undesirable characteristics of currently available grafts discourage the application in CHD patients, but fortunately progresses have been done and promising solutions are underway. The main advantages and disadvantages of materials used for correction of CHD are recapitulated in **Figure 4**.

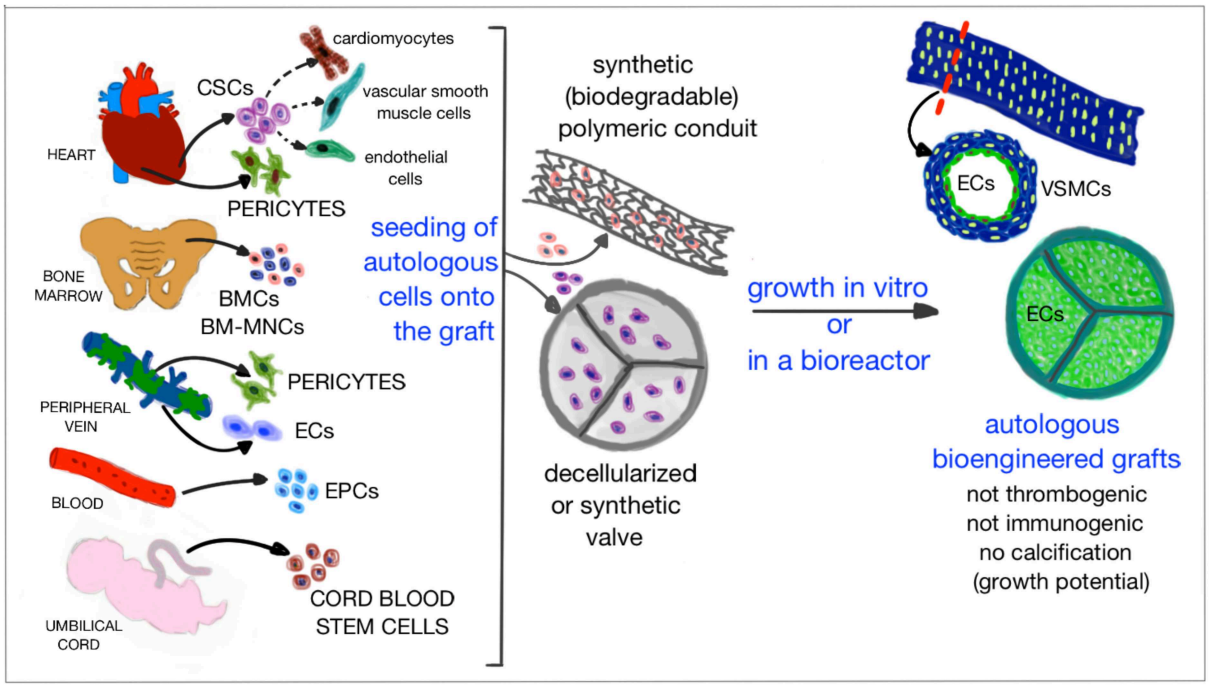

FIGURE 3 | Cartoon illustrating the promising strategy of tissue engineering based on which grafts and materials are combined with patient autologous cells and grown *in vitro* or in a bioreactor, in order to obtain an optimized cellularized graft that lacks of immunogenicity, thrombogenicity, and risk of calcification, while having the potential to grow in parallel with the child growth.

One matter of concern is that the toxicity of chemical agents used during manufacture of prostheses might inhibit the functionalization by autologous cells. A coating with bioactive substances has been proposed to overcome the last problem and promote the process of recellularization *in vivo* Dohmen et al., 2006a; Butcher et al., 2011; Strange et al., 2015. Moreover, the use of glutaraldehyde has been challenged because of concerns about the promotion of calcification. Calcification of grafts, in particular valves, is a major problem since it can cause the failure of the graft and the occurrence of further surgery. The risk of calcification in children is even higher than in adults, as in young patients the mobilization of calcium, due to bone remodeling, accelerates the calcification process. A possible solution is represented by anti-calcification treatments (Butcher et al., 2011; Pok and Jacot, 2011). It remains unknown if calcification may affect cells used for tissue engineering. This risk may be higher for cells, including MSCs, which have the propensity to differentiate into osteocytes under inductive conditions (Ohata and Ozono, 2014). However, it cannot be excluded that other cell types may not only resist to xenograft manufacture agents but also contrast their pro-calcifying action.

The *CorMatrix*® ECM (CorMatrix Alpharetta, GA), a patch made of decellularized porcine small intestinal submucosa extracellular matrix (SIS-ECM), displays a lot of potential advantages over other materials currently used in pediatric cardiac surgery, such as ease handling and implantability, abundance, low immunogenicity, high mechanical strength, minimal scar formation, recruitment of host cells, remodeling without calcification and possible growth potential (Pok and Jacot, 2011; Kalfa and Bacha, 2013). CorMatrix patches have been successfully used in congenital heart surgery for pediatric cardiac and vascular reconstructions (Scholl et al., 2010; Quarti et al., 2011; Witt et al., 2013). Recently, our group confirmed the great recellularization properties of the CorMatrix, successfully growing cardiac pericytes—isolated from children affected by CHD—onto CorMatrix patches up to 3 weeks in a bioreactor, finally showing the engraftment and viability of cells (Avolio et al., 2015b).

Recently, scaffolds have been fabricated using synthetic *biodegradable polymers*, such as polyglicolyc acid (PGA), poly-L-lactic acid (PLLA) and polycaprolactone (PCL). On one hand, the advantages of using these polymers are a limitless supply, ease of shaping, and the possibility to combine the concentrations of the polymers to generate scaffolds that meet the compliance specifications of the environment in which they have to be introduced. But on the other hand, the degradation process by hydrolysis will affect the mechanical properties over time. The degradation rates have been estimated in 2–3 months for PGA and 2 and 3 years for PLLA and PCL, respectively. Although some concerns have been reported about the cytotoxicity of the products of the degradation process (Butcher et al., 2011; Dean et al., 2012; Fallahiarezoudar et al., 2015), these materials are currently the most used in tissue-engineering procedures for the construction of autologous grafts made by the patient's own tissue.

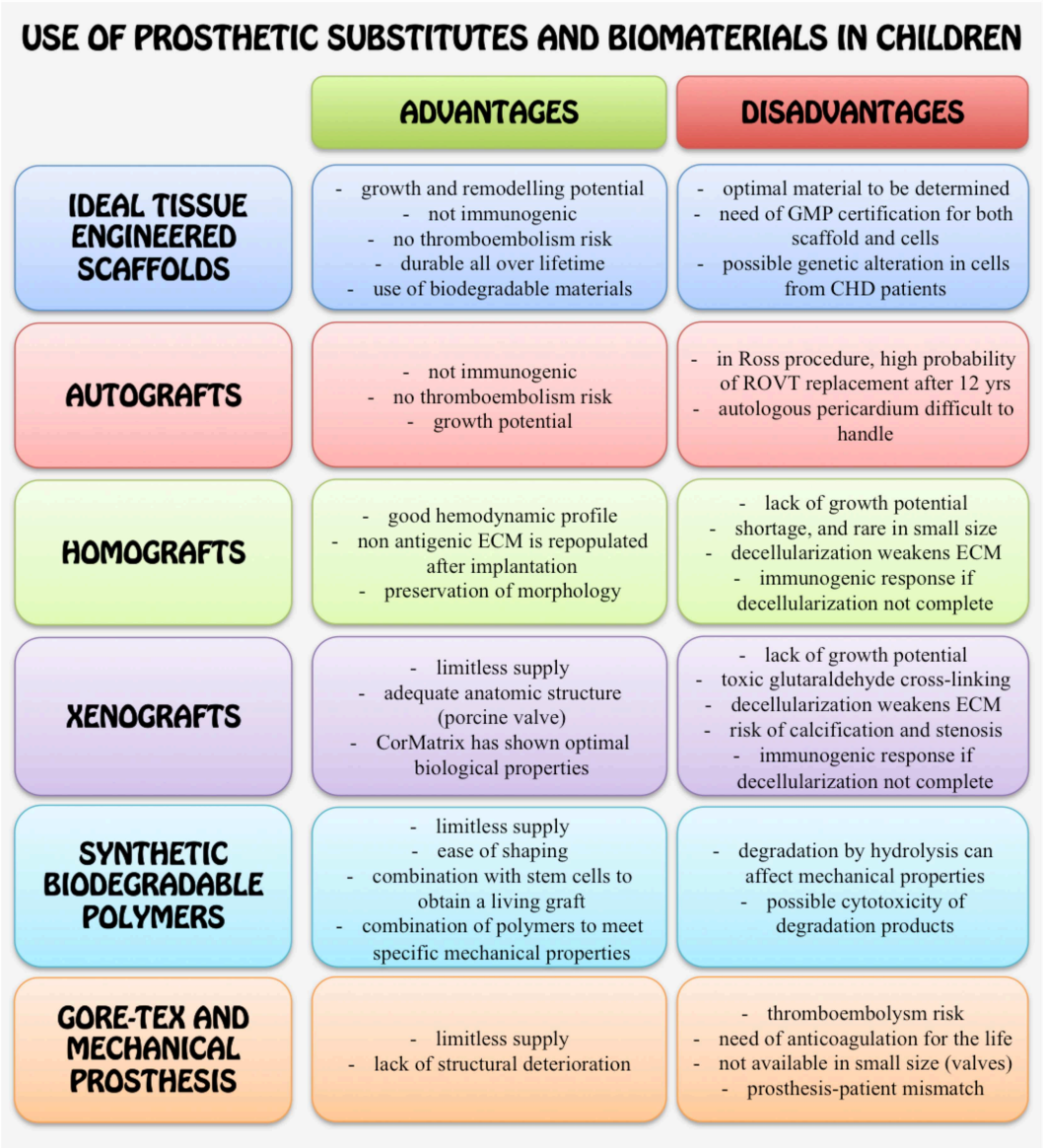

FIGURE 4 | Schematic cartoon summarizing the main advantages and disadvantages of using the different synthetic or biological materials and grafts for surgical correction of CHD in children.

Methods of Cell Seeding and Growth of Tissues *In Vitro*

During the generation of a bioengineered graft, not only the selection of the proper cell type and the choice of a physiological culture method are crucial in order to achieve good results, but also cell seeding is one of the key issues. How to seed cells efficiently and uniformly, especially in the inner parts of scaffolds with pores, and with no impairment to the cells, has been one of the major challenges for tissue engineering.

Since from the beginning, *static seeding* has been the most frequently used because of its simplicity and the ease to be performed within a common cell culture laboratory, since it does not require sophisticated equipment. However, the efficiency of static seeding is always low, and the distribution of the cells throughout the scaffold is not uniform, with the inner part of the scaffold holding few cells, even if excellent biocompatible scaffolds with big pores are used. As a consequence, only the superficial layer of the scaffold will be formed by the regenerated tissue (Dai et al., 2009).

One important factor that limits the penetration of cells inside the scaffold is represented by air in the pores, which explains why static seeding cannot reach satisfactory results in term of scaffold repopulation. Different solutions have been considered to overcome this bottleneck.

In *centrifugation seeding*, a moderate centrifugal force is applied during the seeding process, to facilitate the penetration

of cells in the central part of the scaffold (Godbey et al., 2004; Roh et al., 2007). However, whether survived cells remain functional after centrifugation has still to be determined. Moreover, during centrifugation the orientation of the scaffold cannot be controlled, thus that the overlapping of material pieces does not allow a homogeneous distribution of cells. Instead, the *low-pressure seeding* uses a vacuum desiccator to create a reduced pressure that draws the air in the materials out by pressure difference, facilitating the penetration of the culture media and cells inside the pores (Torigoe et al., 2007). However, despite the seeding efficiency is increased, the low pressure may affect cell viability and function after seeding, thus additional investigations are required (Dai et al., 2009). In *perfusion seeding*, a continuous cell suspension perfusion is applied through 3D-scaffold pores using bioreactors to assist in cell infiltration and to aid in nutrition (Dai et al., 2009).

A *bioreactor* is a device able to ensure the biological and physiological environment of the heart and the circulatory system. Mimicking the physiological mechanical forces, shear stress and blood flow, the bioreactor can allow the construction of engineered scaffolds that will be functional after transplantation into the patients (Carrier et al., 1999; Hecker and Birla, 2007). When cellularized scaffolds are kept in culture for long time, the tissue grows and reaches a 3D-structure, so that the static culture system is not adequate to guarantee the perfusion of culture media uniformly. A solution to this problem comes from the dynamic bioreactors, which can maintain viability of tissue-engineered organs by using both mechanical and biochemical conditioning. Apart the basic requirements of cell cultures—which include the control over the time of dissolved O_2 and CO_2, pH, temperature and nutrients concentration—a dynamic bioreactor can provide the control of flow waveform and physiological pressure, keeping culture medium under the pulsatile flow that enables the perfusion of the media inside the entire graft and simulates at the same time the *in vivo* pressure and heart rate (Carrier et al., 1999; Hecker and Birla, 2007).

Preclinical Studies and Clinical Application of Tissue Engineered-grafts

Tissue engineering-based grafts have been tested in small and large animal models to assess their safety and feasibility before translation to clinics. So far, BMMNCs and ECs have been frequently selected for preclinical studies of tissue engineering applications, probably due to the relative ease of harvesting BM aspirates and peripheral veins compared, for example, to samples of cardiac source. For the sake of brevity and clarity, main animal studies are summarized in **Table 1**. During these procedures, scaffolds were firstly seeded with cells *in vitro*, and secondly implanted into the animals. The results obtained with animals encouraged the test in humans.

Positive results have been obtained also from clinical studies involving the application of tissue engineering-based grafts in children affected by CHD, although they are still limited to few reports. In 2001, the transplantation of a tissue engineering-based graft on a 4-years-old girl with univentricular heart and pulmonary atresia was recorded. Autologous cells were isolated from a peripheral vein and seeded onto a biodegradable tubular scaffold composed by PLLA/PCL reinforced with PGA, designed in order to degrade within 8 weeks. Seven months after implantation, the conduit was not occluded and there was no evidence of aneurysm (Shin'oka et al., 2001). Two years later the same group reported other clinical cases of children with CHD who received a biodegradable tissue engineering-based graft (PLLA/PCL, designed to be degraded in 3–5 years) seeded with total BMCs or selected BMMNCs. Again, the results were positive, with no stenosis documented in the grafts (Matsumura et al., 2003a). The successful transplantation of a vascular biodegradable conduit (PLLA/PCL reinforced with PGA) seeded with saphenous vein derived cells was recorded in a 12-years-old during the Fontan operation (Naito et al., 2003). Later, the clinical results of the application of tissue engineering-based grafts seeded with autologous BMCs in CHD patients (25 out of 42 patients recruited were <7 years) were published (Shin'oka et al., 2005). The scaffolds were made by PLLA/PCL and PGA, degradable in 2 years. The results were positive, without thrombosis, stenosis or obstruction of the grafts after a median of 16 months; grafts remained patent and their diameter increased in size (Shin'oka et al., 2005). More recently, a similar study was conducted in patients with single ventricle physiology (17 out of 25 patients recruited were <7 years), in which PLLA/PCL/PGA grafts seeded with autologous BMMNCs were implanted as extracardiac cavopulmonary conduits. Four out of 25 patients presented graft obstruction that required angioplasty, but all the other patients experimented a good recovery without failure of the graft (Hibino et al., 2010).

Despite these first encouraging results, the performance of TE-grafts in CHD children still needs to be investigated, since a main limitation of the studies performed so far is the lack of a long term follow-up. Nevertheless, application of tissue engineering-based grafts to the correction of CHD is still a new field of study, which will be extensively expanded in the coming future.

Future Perspectives

The choice of the appropriate cell type to use for cell therapy is of crucial importance to reach the desired therapeutic goal. In the future, cell types able to guarantee on the one hand the regeneration of the missing or lost tissue, and on the other hand the parallel remodeling of the extracellular matrix in order to provide the new generated tissue with the ability to adapt with the anatomy of the growing cardiovascular system, might be privileged; also, combinatory cell products may represent an interesting and safe option. But, while there is space for improvement in the characterization and quality of the cell product, the field of regenerative medicine for treatment of CHD urgently requires tissue engineering solutions, as dispersed cells are not ideal to reconstruct valves and conduits. To date, one major limitation in the use of prosthetic devices is the need of replacement. Research is currently underway to provide biologically compatible solutions. While there is a remarkably growing interest in possibilities offered by cell therapy and tissue engineering, the clinical application in newborns and children is still in its pioneering phase.

TABLE 1 | Preclinical studies with tissue-engineered grafts.

Study	Animal model	Cells seeded	Scaffold used	Outcome
Matsumura et al., 2003b	Dog	Allogenic BMMNCs	Copolymer of LA/CL covered by PLLA	TE-grafts were implanted into the vena cava. After up to 8 weeks, no stenosis was observed and cells on the grafts expressed endothelial and VSMc markers
Vincentelli et al., 2007	Lamb	Allogenic BMMNCs or MSCs	Decellularized porcine pulmonary conduits	TE-grafts were implanted into the pulmonary artery. After 4 months, both the valves were recolonized and re-endothe-lialized. BMMNC-valves were thicker and showed inflammatory cell infiltration, while MSC-valves displayed extracellular matrix and cell disposition close to those of native pulmonary valves
Brennan et al., 2008	Lamb	Autologous BMMNCs	PGA scaffolds covered by LA/CL	TE-grafts implanted as inferior vena cava (IVC) interposition grafts. After 6 months, grafts were patent and increased in volume, with no evidence of aneurysmal dilatation. They were histologically comparable to the native IVC
Roh et al., 2010	SCID/beige mice	Xenogenic human BMMNCs	PGA scaffolds covered by LA/CL	TE-grafts were implanted as inferior vena cava interposition grafts. After 24 weeks the original scaffold was degraded and substituted by organized layers of ECM, endothelial and smooth muscle cells, resembling the native IVC
Sutherland et al., 2005	Sheep	Autologous BM-MSCs	PGA/PLLA	The pulmonary valve was resected and TE-valve was implanted into the pulmonary artery. After 4 and 8 months grafts were histologically comparable to the native valve
Shinoka et al., 1995	Lamb	Allogenic ovine artery fibroblasts and ECs	PGA leaflets	The right posterior leaflet of the pulmonary valve was resected and replaced with a TE-valve leaflet. Absence of stenosis. Development of ECM with appropriate cellular architecture
Dohmen et al., 2006b	Sheep	Autologous ECs from jugular vein	Decellularized valve	Scaffold was implanted into the RVOT. After 6 months, there was no calcification, and histologically ECs and fibroblasts were observed
He et al., 2010	Rat	Xenogenic human skeletal muscle pericytes	Poly(ester-urethane) urea scaffolds	TE-grafts were implanted end-to-end into the abdominal aorta. After 8 weeks, pericytes evenly populated the graft. TE-grafts presented extensive tissue remodeling with organized layers of endothelial and smooth muscle cells, and collagen and elastin, resembling the native arterial conduit

Cell therapy products or tissue-engineered products are classified as advanced therapy medicinal products (ATMP) that need to be authorized through centralized procedures. In a tissue-engineered product both components (the scaffold and cell product) must be GMP grade and the combination of the two should be also GMP certified. This includes the manufacture and expansion of cells in the scaffold prior implantation, demonstration of conserved mechanical properties and safety/efficacy in adequate animal models. Therefore, it is likely that the first products to become available for clinical experimentation will be those made by clinical grafts containing cells that have been already tested in clinical trials. In term of safety, it should be kept in mind that CHD is increasingly acknowledged to be associated with genetic polymorphisms. Whether alterations in the genetic program that controls the proper formation of the heart may influence stem cell behavior remains unknown and may represent an additional challenge for the progress of the field.

Advancements in diagnostic screening allow an early diagnosis of CHD and set the basis for guiding the best reconstructive strategy as illustrated in **Figure 5**. In the first scenario, cells are obtained prenatally or at birth with minimally invasive procedures, expanded, incorporated in scaffolds ready for use in definitive surgical correction without need of palliative procedures. Induced pluripotent cells could be ideally generated to this purpose. Alternatively, cord cells have a potential for cases that do not require an immediate intervention at birth. In the second scenario, when diagnosis is made post-natally, stem cells from remnants of palliative surgery could be expanded *in vitro* and combined with biomaterials using bioengineering techniques; afterwards, the resulting cellularized graft will be surgically implanted into the diseased heart at the occasion of a second open-chest surgery, helping the surgeon in performing the reconstructive procedures required to manage complex CHD. Looking at the best cell type, the use of stem cells from cardiac source may give the advantage of a physiological commitment to originate all the 3 major cell types of the heart: cardiomyocytes, ECs and VSMCs. Additional cells with complementary activities, namely vascular cells and pericytes could be added in a combinatory approach.

In conclusion, the potential of tissue engineering may provide in the next future definitive solutions for correction of CHD in newborns and children, opening new avenues with immense therapeutic benefits.

Author Contributions

EA: reviewed the literature, drafted the manuscript and prepared the figures; MC: critically revised the manuscript; PM: reviewed

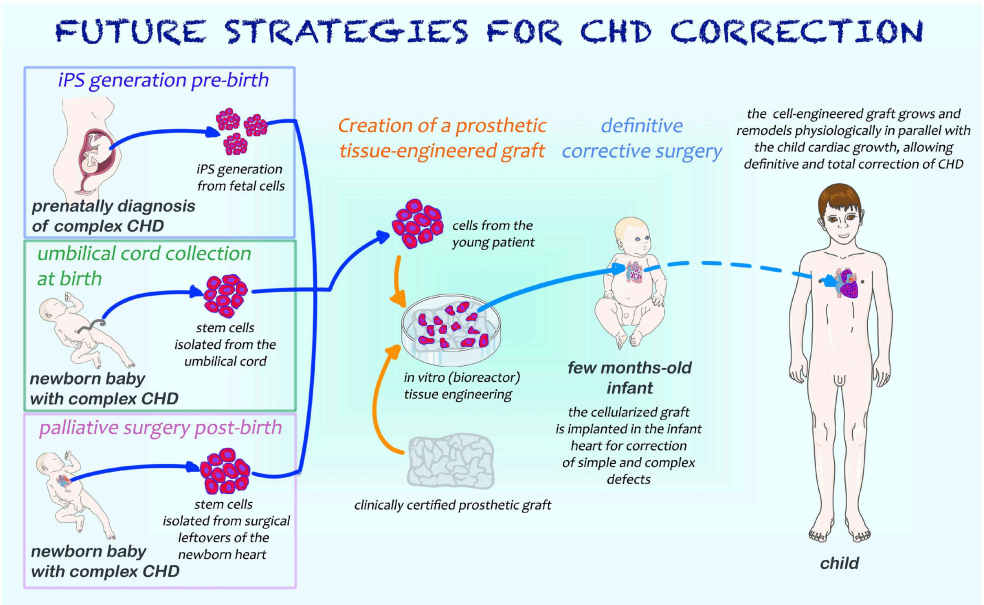

FIGURE 5 | Cartoon illustrating possible future strategies for the surgical management of newborns with CHD. If CHD is diagnosed prenatally, foetal cells may be harvested and iPS generated; as an alternative, umbilical cord stem cells can be isolated at the time of birth. When diagnosis of CHD is made after birth or in babies who require a palliative surgical operation soon after birth, stem cells may be isolated from surgical cardiac leftovers. All these types of cells will allow the generation of a tissue-engineered graft endowed with growth and remodeling potential, necessary for the definitive correction of cardiac defects.

the literature, drafted and critically revised the manuscript. All the authors have approved the final submission of the manuscript.

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Intractable diseases treated with intra-bone marrow-bone marrow transplantation

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Bone marrow transplantation (BMT) is used to treat hematological disorders, autoimmune diseases (ADs) and lymphoid cancers. Intra bone marrow-BMT (IBM-BMT) has been proven to be a powerful strategy for allogeneic BMT due to the rapid hematopoietic recovery and the complete restoration of T cell functions. IBM-BMT not only replaces hematopoietic stem cells (HSCs) but also mesenchymal stromal cells (MSCs). MSCs are multi-potent stem cells that can be isolated from bone marrow (BM), umbilical cord blood (UCB), and adipose tissue. MSCs play an important role in the support of hematopoiesis, and modify and influence the innate and adaptive immune systems. MSCs also differentiate into mesodermal, endodermal and ectodermal lineage cells to repair tissues. This review aims to summarize the functions of BM-derived-MSCs, and the treatment of intractable diseases such as rheumatoid arthritis (RA) and malignant tumors with IBM-BMT.

Keywords: intra-bone marrow-bone marrow transplantation, mesenchymal stem cell, rheumatoid arthritis, malignant tumors, autoimmune diseases

INTRODUCTION

Mesenchymal stromal cells (MSCs) are multi-potent progenitor cells mainly isolated from bone marrow (BM) (Campagnoli et al., 2001), adipose tissue (Zuk et al., 2001), and the umbilical cord (UC) (Erices et al., 2000). MSCs have been shown to differentiate into osteoblasts, adipocytes (Dominici et al., 2006), cardiomyocytes (Makino et al., 1999), and pancreatic islets (Tang et al., 2004). Furthermore, MSCs have the ability to migrate to injured tissue of liver (van Poll et al., 2008) and heart (Yokokawa et al., 2008). Adipose tissue and BM are the most readily available sources of MSCs because they are easy to harvest, and there are no ethical concerns. BM-derived MSCs (BMMSCs) have a higher degree of commitment to differentiate into chondrogenic and osteogenic lineages than adipose tissue-derived MSCs (Gimble et al., 2007), although there appears to be no difference between adipose tissue-derived MSCs and BM MSCs in terms of immunoregulatory functions and support of hematopoiesis (Poloni et al., 2012). On the other hand, BM MSCs modulate the immune response, suppress allogeneic T cell responses, and prevent the development of graft-versus-host disease (GVHD) (English, 2013).

BM transplantation (BMT) is useful for treating hematopoietic disorders, allogeneic BMT also being used to treat autoimmune diseases (ADs) (Nishimura et al., 1994). Intra-bone marrow-bone marrow transplantation (IBM-BMT) has been proven to be the most effective approach to treating allogeneic BMT, since IBM-BMT can replace not only hematopoietic stem cells (HSCs) but also BMMSCs Thus hematopoietic recovery is rapid, and no GVHD develops even if whole BM cells are injected (Kushida et al., 2001; Ikehara, 2003). In this review, we focus on rheumatoid arthritis (RA) and malignant tumors treated with IBM-BMT.

IMMUNOREGULATORY FUNCTIONS OF BMMSCs

BMMSCs have been reported to have the ability to modify and influence almost all the cells of the innate and adaptive immune systems mediated by BMMSC soluble factors, including IL-6, M-CSF, IL-10, TGFβ, HGF, and PGE2 (Aggarwal and Pittenger, 2005; Beyth et al., 2005; Ramasamy et al., 2007). The adaptive immune system, which is composed of T and B lymphocytes, generates specific immune responses to pathogens with the production of memory cells. BMMSCs modulate the function of dendritic cells (DCs), indirectly regulate T and B cell activity, and delay or prevent the development of acute GVHD (Zhang et al., 2009). BMMSCs have also been shown to suppress the differentiation of DCs and their function during allogeneic islet transplantation (Urban et al., 2008; Aldinucci et al., 2010). BMMSCs strongly inhibited the maturation and functioning of monocyte-derived DCs by interfering selectively with the generation of immature cells via inhibitory mediator of MSC-derived PGE2 (Lee et al., 2006). PGE2 has been identified as one of the candidates responsible for T cell inhibition by BMMSCs, and may have an immunostimulatory role by facilitating Th1 differentiation and expanding the Th17 T cell population (English et al., 2009; Yao et al., 2009). The expression of PGE2 was shown to be upregulated by IFNγ and TNFα in the BMMSCs for immunomodulatory function (English et al., 2007). BMMSCs can inhibit the cytotoxic effects of antigen-primed cytotoxic T cells by suppressing the proliferation and activity (Zhao et al., 2005) via the inhibition of the nuclear translocation of nuclear factor-kappa B (Matsuda-Hashii

et al., 2004). BMMSCs have been shown to alter the NK cell phenotype and suppress proliferation of NK cells via the secretion of TGFβ1 and PGE2, and via cytotoxicity against HLA class I-expressing targets (Aggarwal and Pittenger, 2005; Sotiropoulou et al., 2006; Ryan et al., 2007; Uccelli et al., 2008). BMMSCs have also been shown to inhibit the proliferation of B cells when stimulated with anti-CD40L and IL-4 (Glennie et al., 2005). One report has suggested that allogeneic BMMSCs inhibit the activation, proliferation and IgG secretion of B cells in a BXSB mouse model of human systemic lupus erythematosus (Deng et al., 2005).

Allogeneic BMMSCs are effective in the treatment of murine models of human disease (Zappia et al., 2005; Ding et al., 2009; Fiorina et al., 2009). BMMSCs were shown to be able to secrete regulatory cytokines that affect regulatory T cells, and to modulate the immunological dysregulation observed in antibody producing B cells and cytotoxic NK cells in the NOD mouse (Anderson and Bluestone, 2005). BMMSCs promote the endogenous repair of pancreatic islets and renal glomeruli in a streptozotocin-induced diabetic mouse model (Lee et al., 2006). Co-infusion of BMMSCs and BM cells was shown to inhibit the beta cell-specific T cell proliferation and to restore insulin and glucose levels (Urban et al., 2008). BMMSCs secrete many cytokines and growth factors such as HGF, which shows anti-apoptotic activity in hepatocytes and plays an essential part in the regeneration of the liver (Trim et al., 2000; Matsuda-Hashii et al., 2004). BMMSCs have also been shown to protect against experimental liver fibrosis in CCl4-induced rats (Zhao et al., 2005), and to suppress CD3 T-cell proliferation in collagen-induced arthritis (Schurgers et al., 2010).

In mammals, there are seven sirtuin family members, named Sirt1-7. Sirtuins plays a critical role in the regulation of fundamental biological responses to nutritional and environmental stimuli in each subcellular compartment (Blander and Guarente, 2004; Imai and Guarente, 2010). Sirt1 is a class III protein deacetylase, and Sirt1 activity can be regulated through NAD^+. Sirt1 binds to and deacetylates a number of important transcription factors—such as peroxisome proliferator-activated receptor gamma (PPARγ), PPARα, PPAR gamma coactivator 1 alpha (PGC-1α), and the forkhead box, subgroup O (FOXO) family of transcription factors—to drive metabolic responses such as insulin secretion, gluconeogenesis, and fatty acid oxidation (Haigis and Sinclair, 2010). Some reports indicate that Sirt1 promotes osteogenesis and decreases adipogenesis of BMMSCs *in vitro* (Tseng et al., 2011; Peltz et al., 2012; Puri et al., 2012).

Sirt1 deacetylates β-catenin to regulate differentiation of MSCs in MSCs specific Sirt1 knock-out mice (MSC KO) (Simic et al., 2013). Moreover, Sirt1 has been shown to directly downregulate *Sost* gene expression, and promote bone formation in the treatment of osteoporosis (Cohen-Kfir et al., 2011). One report has shown that CD8 T cell differentiation is regulated by basic leucine zipper transcription factor, ATF-like (BATF), which is a member of the AP-1 family, via Sirt1 expression, BATF deficiency inducing high levels of Sirt1 expression in memory CD8 T cells but not in naive CD8 T cells (Kuroda et al., 2011).

IBM-BMT

We reported that MRL/lpr mice possess abnormal radioresistant stem cells and have provided impressive evidence regarding the origin of ADs in this strain (Ikehara et al., 1989). BMT plus bone graft, which can recruit donor stroma cells, can prevent the recurrence of ADs (Ishida et al., 1994). However, allogeneic BMT + bone grafts failed to treat ADs in MRL/lpr mice, because these mice become more radiosensitive after the onset of lupus nephritis. Moreover, our previous reports showed that stroma cells can be trapped in the liver when BM cells are injected via the portal vein. Thus, directly injecting whole BM cells into the BM, as in IBM-BMT, has been shown to be a powerful strategy for the treatment of ADs in MRL/lpr mice. IBM-BMT, which not only replaces HSCs but also MSCs, has been proven to be the best method for allogeneic BMT: (1) hematopoietic recovery is rapid because the MSCs directly home to the bone cavity, (2) the restoration of T cell functions is complete even in donor-recipient combinations across the MHC barriers, and (3) no graft failure occurs even if the radiation dose is reduced (Kushida et al., 2001). Moreover, IBM-BMT of young marrow cells reversed the reduction of pro-B cells and pre-B cells. The frequency of follicular-B cells in the IBM-BMT group was significantly increased compared to the old group (Hida et al., 2010). We have already used IBM-BMT to successfully treat ADs, osteoporosis, diabetes, Alzheimer's disease, and for the induction of tolerance for organ transplantation (Takada et al., 2006; Guo et al., 2008; Kushida et al., 2009; Li et al., 2009, 2010) (**Table 1**).

BM cells mainly include HSCs and MSCs. MSCs are essential for supporting hematopoiesis in the BM. HSCs can normally proliferate in major histocompatibility complex (MHC)-compatible MSCs even in allogeneic microenvironments. Because the BMCs are directly injected into bone, IBM-BMT circumvents the risk of MSCs being trapped in the lung and liver. And because both MSCs and HSCs are transplanted, hematopoiesis can be rapidly restored. Moreover, IBM-BMT can prevent the risk of graft rejection, even with the use of a mild conditioning regimen (Kushida et al., 2001).

IBM-BMT FOR TREATMENT OF RA

RA is an AD that results in a chronic, systemic inflammatory disorder that may affect many tissues and organs. RA primarily affects joints, but it also affects other organs such heart, kidney, and blood vessels (Turesson et al., 2003). Its pathophysiology indicates that TNFα drives synovial inflammation and joint destruction. The synovial cells include both fibroblast-like and macrophage-like synoviocytes. Fibroblast-like synoviocytes show abnormal behavior in RA (Scott et al., 2010). About 50% of RA is caused by genetic abnormalities (van der Woude et al., 2009). The classification criteria for RA by the American College of Rheumatology (2010), and the treatment options, are summarized in the work by Scott et al (Scott et al., 2010). Here we talk about stem cell therapy for the treatment of RA in basic experiments and clinical applications.

SKG/Jcl mice are a murine model for RA. BM cells of C57BL/6J mice were transplanted into SKG/Jcl mice using IBM-BMT, and the hematolymphoid cells in the recipient mice were reconstituted by donor-derived cells. There was no evidence of arthritis

Table 1 | IBM-BMT treatment of various diseases and induction of tolerance for organ transplantation.

Authors	Animal model	Effect of IBM-BMT
Li et al., 2012	Mouse	Improve renal function
Zhang et al., 2012	Mouse	Prevention of leukemia
Shi et al., 2011	Mouse	Diminish risk of GVHD
Feng et al., 2010	Mouse	Prevention of premature ovarian failure
Li et al., 2009	Mouse	Amelioration of cognitive ability
Kushida et al., 2009	Mouse	Prevention of rheumatoid arthritis
Okazaki et al., 2008	Mouse	Liver transplantation
Miyake et al., 2008	Mouse	Prevention of GVHD
Abraham et al., 2008	Mouse	Prevention of type 2 diabetes
Guo et al., 2008	Rat	Long-term donor specific tolerance in cardiac allograft
Feng et al., 2007	Mouse	Prevention of osteoporosis and hypogonadism
Koike et al., 2007	Mouse	Suppression of growth of colon cancer cells
Ikebukuro et al., 2006	Mouse	Tolerance induction in allogeneic pancreatic islets
Kaneda et al., 2005	Rat	Induction of tolerance for lung transplantation
Takada et al., 2006	Mouse	Prevention of senile osteoporosis
Taira et al., 2005	Rat	Prevention of type 1 diabetes
Nakamura et al., 2004	Mouse	Prevention of GVHD
Esumi et al., 2003	Rat	Induction of tolerance for allogeneic leg transplantation
Ichioka et al., 2002	Mouse	Prevention of senile osteoporosis

in the SKG/Jcl mice at 12 months after transplantation. Moreover, IBM-BMT has been shown to normalize the percentages of Treg (Foxp3+/CD4+) cells, the percentages of receptor activator of NF-kB ligand+ cells on the CD4+ T cells and the serum levels of TNFα, IL-1, and IL-6. One report demonstrated that IBM-BMT is a viable method of immunological manipulation that suppresses the severe joint destruction and bone absorption in SKG/Jcl mice and lends further credence to the use of this methodology in humans with intractable RA (Kushida et al., 2009). Human UC-derived MSCs have been discussed as a possible treatment for RA in the clinical setting. TNFα and IL-6 decreased and CD4+ CD25+ Foxp3+ T cells increased, in active RA patients after UC-derived MSCs were infused, and the UC-derived MSCs survived for 3–6 months, suggesting that treatment with MSCs would benefit RA patients (Wang et al., 2013). Expression of IL-17, IL-6, and TNFα were inhibited when allogeneic UC-derived MSCs were cultured with peripheral blood mononuclear cells (PBMCs) from RA patients, suggesting that MSCs can prevent the expression of these cytokines and that they have therapeutic potential in the treatment of RA (Wang et al., 2012).

MALIGNANT TUMORS TREATED WITH IBM-BMT + THYMUS TRANSPLANTATION (TT)

Donor lymphocyte infusion (DLI) is a useful method for the treatment of malignant tumors, but it also induces GVHD. However, IBM-BMT has been shown to prevent not only graft failure but also GVHD in animals, even when the radiation dose is reduced (Nakamura et al., 2004). Thus, IBM-BMT plus DLI were used to treat malignant tumors (fibrosarcomas) induced by a tumor cell line (methA). DLI ($CD4^-$ spleen cells) can prevent GVHD, but the tumor growth was not suppressed, indicating that CD4+ cells play important roles in graft-versus-tumor (GVT) and GVHD. Our previous results showed that IBM-BMT plus DLI ($CD4^-$lymphocytes) suppressed not only GVHD but also tumor growth (Suzuki et al., 2005). Moreover, the combination of DC, IBM-BMT and DLI showed even better results than the combination of IBM-BMT and DLI in the treatment of solid tumors (Mukaide et al., 2007).

The thymus regulates the production, proliferation and functions of T cells. BMT + TT has been shown to be useful in the treatment of ADs in the MRL/Lpr mouse, because the allogeneic T cells newly-developed by TT are naïve T cells, which show less Fas expression and more resistance to apoptosis than the activated memory T cells with their high Fas expression. We found that the combination of allogeneic IBM-BMT + adult TT from the same donor is effective in mice with solid tumors, as it can induce high thymopoiesis, preserving strong GVT effects without inducing a severe graft-versus-host reaction (GVHR). Meth A sarcoma cells were subcutaneously inoculated into mice, and IBM-BMT + adult TT was then used to treat these mice when the tumor had grown to 5 mm. In tumor-bearing mice, tumor growth was more strongly inhibited by IBM-BMT + adult TT than by IBM-BMT alone. The numbers of $CD8^+$ T cells that infiltrated the tumors, and the number of apoptotic tumor cells, both significantly increased in the mice treated with IBM-BMT + adult TT. IBM-BMT + adult TT prevented tumor development with mild GVHR resulting from the induction of high thymopoiesis and a strong GVT effect in the tumor-bearing mice. The number of $CD4^+$ $FoxP3^+$ cells was lower in the mice treated with IBM-BMT + adult TT than in those treated with IBM-BMT alone. Furthermore, the numbers of $CD8^+$ cells infiltrating the tumor and the levels of IFN-γ were higher in the mice treated with IBM-BMT + adult TT than in those treated with IBM-BMT alone (Miyake et al., 2009). Although T regs have been reported to suppress the GVHR induced by $CD4^+$T cells, they did not reduce the GVT induced by $CD8^+$ T cells (Edinger et al., 2003). Tumors were suppressed to a greater extent as a result of the increased $CD4^+$ and $CD8^+$ T cells and decreased number of $Gr\text{-}1^+/CD11b^+$ myeloid suppressor cells and $Foxp3^+/CD4^+$ T regs, Moreover, the production of $CD62L^-CD44^+$ effector memory T cells and IFN-γ were also higher (Zhang et al., 2011).

IBM-BMT seems to be better than co-transplantation of HSCs and cultured MSCs, mainly because the number of functional MSCs may drop after being cultured *in vitro*, and cultured MSCs also are trapped by the liver and lung in the case of IV-BMT. Umbilical cord blood (UCB) can also be used a source of stem cells for transplantation, although the numbers are generally insufficient to allow this to be used as a general source. IBM

thus appears to be the best choice for allogeneic transplantation, despite the limited number of stem cells that can be directly transplanted into the bone cavity. In conclusion, IBM-BMT can efficiently transplant both HSCs and MSCs, is useful to treat intractable diseases such as RA and malignant tumors, and in the future may be useful for treating various intractable diseases.

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Recruitment of bone marrow-derived cells to periodontal tissue defects

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Bone marrow-derived cells (BMCs) are considered to be a major source of mesenchymal stem cells (MSCs) in adults and are known to be effective in periodontal tissue regeneration. However, whether endogenous BMCs are involved in periodontal tissue repair process is uncertain. We therefore created periodontal tissue defects in the buccal alveolar bone of mandibular first molars in bone marrow chimeric mice, and immunohistochemically examined the expression of stromal cell derived factor-1 (SDF-1) and the mobilization of BMCs. We found that SDF-1 expression was increased around the defects at as early as 1 week after injury and that BMCs were mobilized to the defects, while GFP+/CD45+ were rarely observed. Fluorescence-activated cell sorting (FACS) analysis demonstrated that the number of platelet-derived growth factor receptor (pdgfr) α+/Sca-1+ (PαS) cells in the bone marrow decreased after injury. Taken together, these results suggest that BMCs are mobilized to the periodontal tissue defects. Recruitment of BMCs, including a subset of MSCs could be a new target of periodontal treatment.

Keywords: bone marrow chimeric mice, periodontal defects, mesenchymal stem cells, recruitment, SDF-1

INTRODUCTION

Periodontal disease is a bacterially induced chronic inflammatory disease that destroys the tooth-supporting tissue and is one of the main causes of tooth loss. The inflammation and breakdown of tissue can be prevented by conventional periodontal treatment such as scaling and root planing (tissue debridement). However, the conventional treatment does not regain the tissue that has been lost during the disease process. Recently MSC-like cells have been discovered in periodontal ligament (PDLSCs) (Seo et al., 2004) and extensive studies have been carried out to investigate the potential use of PDLSCs as a therapeutic agent for periodontal regeneration.

Mesenchymal stem cells (MSCs) show multi-differentiation capability and self-renewability *in vitro*. Bone marrow is considered as the one of main source of MSCs. Bone marrow MSCs have attracted attention as donor cells for regenerative therapy, and the efficacy of bone marrow MSCs has been also shown by experiments in the periodontal tissue regeneration. For example, it has been reported that canine periodontal defects can be regenerated from bone marrow MSCs mixed with atelocollagen (Kawaguchi et al., 2004) and that transplanted bone marrow MSCs were observed in regenerated periodontal tissue (Hasegawa et al., 2006). Yang et al. demonstrated that engraftment of bone marrow-derived MSCs with gelatin beads successfully regenerated periodontal tissue in rats (Yang et al., 2010).

Endogenous bone marrow-derived cells (BMCs) including MSCs have been reported to promote repair of the remote tissue by mobilizing into peripheral blood by injury signals, and homing to injured tissues (Mansilla et al., 2006; Alm et al., 2010). Recently, SDF-1 has been reported to involve in the recruitment and engraftment of stem cells in wound sites (Yin et al., 2010). C-X-C chemokine receptor type 4 (CXCR4) is a unique receptor of SDF-1, and is known to express in various cell types, including stem cells (Honczarenko et al., 2006). However, whether the SDF-1/CXCR4 axis is involved in recruitment of BMCs to periodontal wound is not clarified. Particularly, the contribution of endogenous bone marrow MSCs during periodontal tissue repair is not fully understood due to lack of appropriate detection system of MSCs *in vivo*.

A lack of the unique marker is a major obstacle for detection of MSCs *in vivo*. The characteristics of MSCs were confirmed by combination expression of cell surface markers such as CD44, CD73, CD90, and CD105 in a single cell. Besides it must be shown that there is not expression of hematopoietic stem cell marker, CD34 and hematopoietic progeny markers such as CD11b, and CD45. Recently, Morikawa et al. reported the method to isolate MSCs from murine bone marrow without cell culture by cell sorting of pdgfrα (+)/Sca-1 (+) cells (Morikawa et al., 2009).

In this study, we created periodontal defects in the buccal alveolar bone of mandibular first molars in bone marrow chimeric mice and investigated the expression of SDF-1 and the recruitment of bone marrow MSCs during periodontal tissue repair process.

MATERIALS AND METHODS

PREPARATION OF BONE MARROW CHIMERIC MICE

BMCs were isolated from femurs and tibias of GFP transgenic mice as reported previously (Okabe et al., 1997; Sata et al., 2002; Fukuda et al., 2009). In brief, BMCs were hemolyzed with ACK lysing buffer (Lonza, Basel, Switzerland). C57/BL/6 mice (age, 8 weeks; male) were lethally irradiated 9.5 Gy (MBR-1520RB; Hitachi, Tokyo, Japan). Two days after irradiation, unfractionated BMCs (1×10^6 cells/0.3 ml D-PBS) from GFP transgenic mice were intravenously injected into irradiated mice by tail vein puncture. Eight weeks after the transplantation, peripheral bloods were collected from retro-orbital plexus. Replacement ratio of bone marrow was confirmed by fluorescence-activated cell sorting (FACS) aria (BD, Franklin Lakes, NJ, USA). Chimeric mice with a bone marrow substitution rate of over 83% were used in this experiment. All procedures involving the experimental animals were performed in accordance with protocols approved by the local institutional guidelines for animal care of The University of Tokyo and Tokyo Medical and Dental University (0120218A) and complied with the *Guide for the Care and Use of Laboratory Animals* (NIH guidelines 32).

EXPERIMENTAL PERIODONTAL TISSUE INJURY

Under general anesthesia with sodium pentobarbital (40–50 mg/kg, IP), we produced 2.0×1.5 mm periodontal tissue defects in the buccal alveolar bone of mandibular first molars in bone marrow chimeric mice, by removing alveolar bone, periodontal ligament and cementum using a round bar with water cooling under a stereoscopic microscope.

IMMUNOHISTOCHEMICAL STAINING

Before or at 1, 2, 4, 5, and 10 weeks after injury, tissue was fixed with 4% paraformaldehyde, followed by decalcification in 10% ethylenediaminetetraacetic acid (EDTA) solution at 4°C. Tissue sections (5 μ) were immunostained for GFP and SDF-1. Briefly, after deparaffinization, sections were washed with PBS, and were treated with 1% hydrogen peroxide (Wako, Osaka, Japan) in methanol. After washing with PBS, sections were blocked with blocking solution (0.5% goat serum in PBS) at room temperature for 30 min.

Before anti mouse SDF-1 antibody treatment, sections were treated with a M.O.M. Immunodetection Kit (Vector Laboratories Inc., Burlingame, CA, USA). Sections were then treated with either anti-mouse GFP antibody (1: 500 dilution, #47894A; Molecular Probes, Eugene, OR) or anti mouse SDF-1 antibody (1: 1000 dilution, #79014; R&D systems, Minneapolis, MN, USA) or anti-mouse CD45 antibody (1: 200 dilution #550539; BD) for 2 h. After washing with PBS three times, sections were treated with secondary antibodies, either anti-rabbit conjugated with biotin (Dako Japan, Kyoto, Japan) or anti-mouse conjugated with biotin (Dako Japan) or anti-rat Cy3 (1: 200 diluted, #56021; Jackson, West Grove, PA, USA) at room temperature for an hour. After washing with PBS three times, sections were treated with ABC-AP mix (Vector Laboratories Inc.) at room temperature for an hour. Detection was performed using Vector Red at room temperature for 10–20 min. Histological examination was performed under a fluorescence microscope (BZ-8000; Keyence, Osaka, Japan)

FLUORESCENCE-ACTIVATED CELL SORTER (FACS) ANALYSIS FOR IDENTIFICATION OF BONE MARROW MSCs

In the experimental group, BMCs were obtained from murine femurs and tibias 1 week after preparation of periodontal defects. Untreated mice were used as controls. To evaluate the ratio of bone marrow MSCs for all collected cell counts, we conducted at least three experiments using two mice in the experimental and control groups. BMCs were obtained as described previously (Morikawa et al., 2009). Briefly, femurs and tibias were aseptically removed from two mice and bones were crushed with an ice-cold pestle and mortar. Bone chips, including marrow were rinsed with HBSS+ and were digested using collagenase (#032-22364; Wako) for an hour at 37°C. Collected BMCs were hemolyzed and FcR was blocked with anti-mouse CD16/32 (#553142; BD) on ice for 5 min. BMCs ($2–5 \times 10^5$) were multi-stained with CD45.2-APC-eFlour780 (#47-0454; eBioscience, San Diego, CA, USA), TER119-PECy7 (#25-5921; eBioscience), pdgfrα-PE (#12-1401-81; eBioscience), Sca-1-APC (#17-5981-81; eBioscience) (1: 100 dilution, 30 min, on ice) and 7AAD (#559925; BD) (1: 100 dilution, 10 min, on ice). The ratio of MSCs [CD45.2 (−), TER119 (−), 7AAD (−), pdgfrα (+), Sca-1 (+)] in BMCs was evaluated by FACS analysis.

RESULTS

LOCALIZATION OF GFP-POSITIVE CELLS IN PERIODONTAL TISSUE

In order to investigate the localization of BMCs, we prepared bone marrow chimeric mice and examined GFP-positive cells in the periodontium. Both osteoclasts-like multinucleated giant cell in resorption pits of alveolar bone and macrophages in gingival epithelium, which are known to be derived from bone marrow, were positive for GFP (**Figures 1A,B**). In control mice, GFP-positive cells were observed in both periodontal ligament and dental pulp (**Figures 1C–E**). GFP-positive cells in periodontal ligament were mainly observed around blood vessels (**Figure 1D**). GFP-positive cells were rarely observed in alveolar bone or dentin.

TIME-COURSE OBSERVATIONS OF BMCs AROUND PERIODONTAL TISSUE DEFECTS

Experimental periodontal tissue defects were created in bone marrow chimeric mice. Five weeks after injury, GFP-positive cells were observed in the periodontal ligament in both control and experimental groups. In the experimental group, the number of GFP-positive cells increased significantly around the periodontal tissue defects (**Figure 2**). Ten weeks after injury, GFP-positive cells around the periodontal tissue defects decreased to control levels. Meanwhile, no changes were seen during the course of experiment in control tissue. In addition, we performed double staining for SDF-1 and GFP in order to check co-localization of SDF-1 and GFP. Five weeks after injury, SDF-1 expression was ubiquitously seen in periodontal ligament and the number of BMCs in experimental tissues was higher than in controls (**Figure 3**). In control tissue, SDF-1 expression was dominantly observed around blood vessels. A double staining for GFP and CD45 showed limited co-localization of GFP (green) and CD45

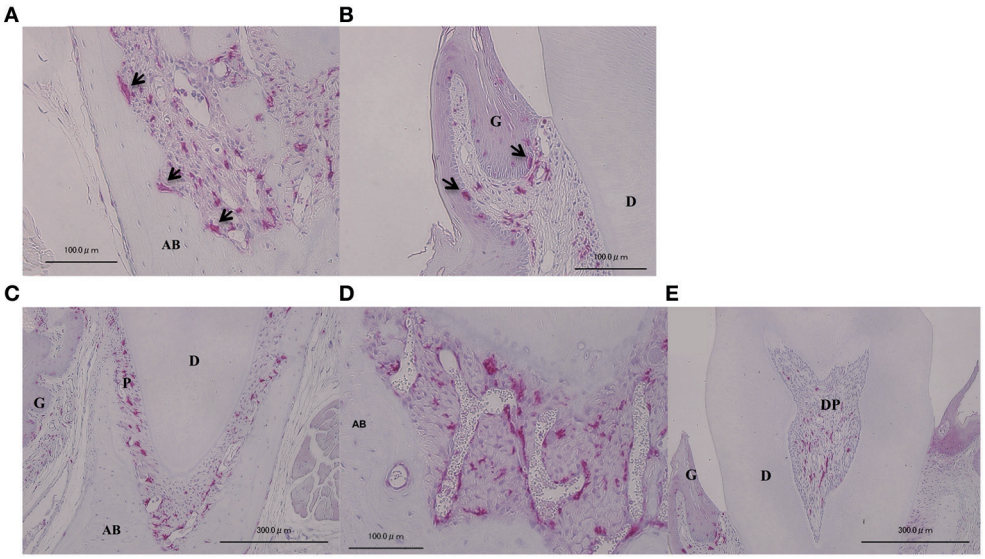

FIGURE 1 | Localization of GFP-positive cells in intact periodontal tissue. Visualization of BMCs *in vivo* on periodontal tissue. Arrows indicate osteoclast-like multinucleated giant cells **(A)** and macrophage-like cells in gingival epithelium **(B)** were GFP-positive, implying that GFP-positive images were confirmed to match BMCs. GFP-positive cells were also observed at the periodontal ligament **(C)**, blood vessels **(D)**, and dental pulp **(E)**. A, multinucleated giant cells in resorption lacunae of alveolar bone; B, gingival epithelium; C, periodontal ligament; D, blood vessels in periodontal ligament; E, dental pulp. G, gingiva; AB, alveolar bone; P, periodontal ligament; DP, dental pulp; D, dentin.

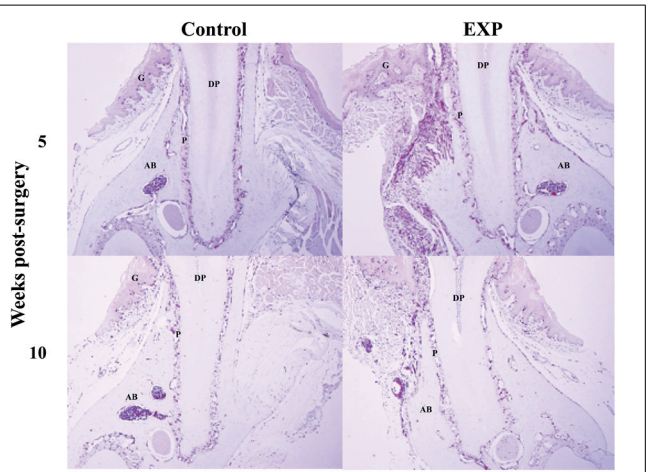

FIGURE 2 | Time-course observations of BMCs around periodontal tissue defects. Experimental periodontal tissue defects were introduced at the buccal surface of mandibular first molar roots in bone marrow chimeric mice. Five weeks after surgery, the number of GFP-positive cells was significantly elevated around the defects. Ten weeks after surgery, GFP-positive cells around the periodontal tissue defects decreased to control levels. G, gingiva; AB, alveolar bone; P, periodontal ligament; DP, dental pulp; D, dentin.

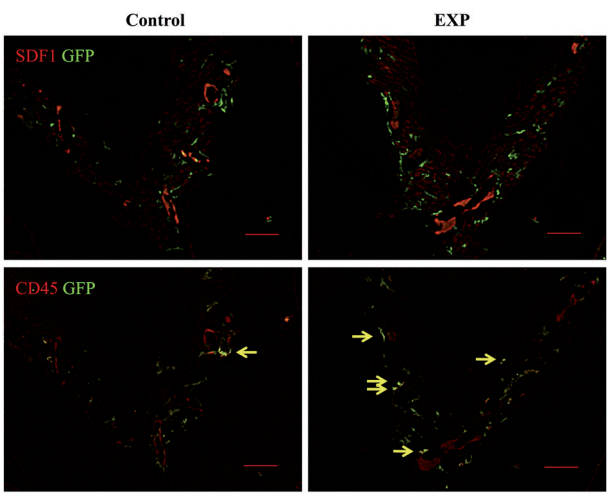

FIGURE 3 | Double staining of GFP and SDF-1/CD45 in periodontal ligaments. In control sites, blood vessel-like structures showed intense staining of SDF-1. GFP-positive cells were observed around SDF-1-positive cells. In experimental sites, SDF-1 expression was ubiquitously detected in periodontal ligament 5 weeks after surgery. The number of GFP-positive cells in experimental tissues was higher than in controls. In experimental tissue, the number of GFP(+)/CD45(+) cells (yellow arrows) was elevated, accounting for 13% of GFP-positive cells ($n = 4$).

(red). In experimental tissue the number of GFP(+)/CD45(+) cells peaked at 13% GFP-positive cells.

TIME-COURSE OBSERVATIONS OF SDF-1 EXPRESSION AROUND PERIODONTAL TISSUE DEFECTS

At 1, 2, and 4 weeks after injury, SDF-1 expression was immunohistochemically evaluated (**Figure 4**). One week after injury, some SDF-1 was observed within the blood vessels of periodontal ligaments in control tissue. In experimental tissue, weak and diffuse SDF-1 staining was observed in the defects and periodontal ligament. After 2 weeks, SDF-1 staining increased markedly and was attenuated by 4 weeks after injury.

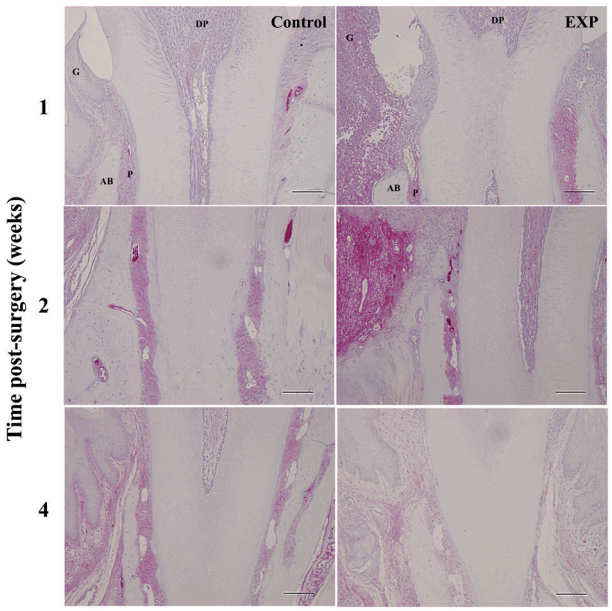

FIGURE 4 | Time-course observations of SDF-1 expression around periodontal tissue defects. In experimental sites, SDF-1 expression was slightly higher at 1 week after surgery when compared with controls. SDF-1 expression peaked at 2 weeks after surgery in experimental tissue. SDF-1 expression decreased to control levels by 4 weeks. G, gingiva; AB, alveolar bone; P, periodontal ligament; DP, dental pulp.

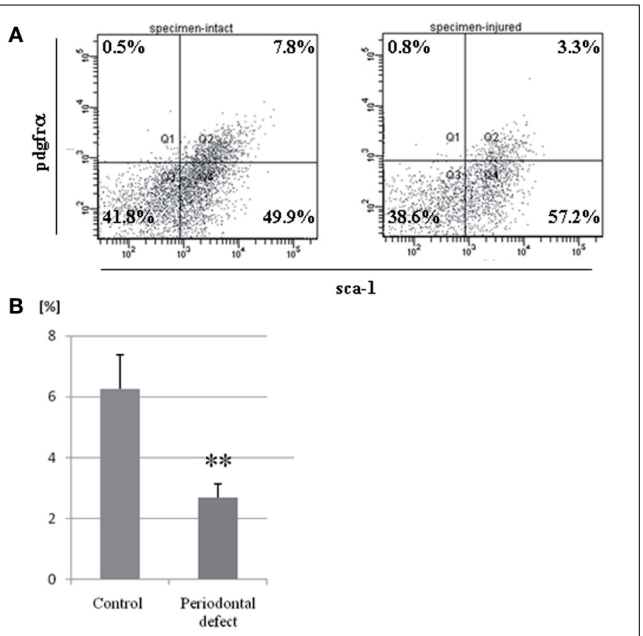

FIGURE 5 | Postoperative changes in MSC population in bone marrow. BMCs were collected from murine femurs and tibias in both the control and experimental groups. **(A)** A representative data from FACS analyses. **(B)** FACS analysis showed that the percentage of MSCs in bone marrow of mice with periodontal defects was significantly lower than in controls. (**$p = 0.01$) Independent experiments were repeated at least five times.

POST-OPERATIVE CHANGES IN MSC POPULATION IN BONE MARROW

One week after injury, BMCs were collected from murine femurs and tibias. Immediately after collecting BMCs, MSC population in bone marrow was evaluated by FACS analysis. FACS analysis confirmed that the percentage of pdgfrα (+)/Sca-1(+) cells in bone marrow of mice with periodontal defects was significantly lower when compared to controls (**Figure 5**).

DISCUSSION

Involvement of BMCs in the physiology and pathology of various tissues, including heart, lung, liver, kidney, skeletal muscle, bone, and blood vessels, has been reported. Some of these studies used bone marrow chimeric mice and successfully tracked BMCs. In order to determine roles of endogenous BMCs in periodontal tissue, we created bone marrow chimeric mice. Both osteoclast-like multinucleated giant cells at the alveolar bone and macrophages in gingival connective tissue, known to be of bone marrow origin, were positive for GFP staining (**Figures 1D,E**), implying BMCs were successfully tracked. However, we should be aware of possibility that recipient bone marrow cells may fuse with donor cells, acquiring such phenotypes in periodontal tissue. GFP-positive cells were observed in both the periodontal ligament and dental pulp (**Figures 1A,B**). To our knowledge, this is the first time that the presence of endogenous BMCs has been demonstrated in naive murine periodontal ligament, at least by observation of bone marrow chimeric mice. In addition, these cells were mainly observed around blood vessels in periodontal ligament (**Figure 1C**). McCulloch reported that stem cell-like cells with a slow rate of cell proliferation were located in paravascular sites in murine periodontal ligament (McCulloch, 1985). Chen et al. reported that putative stem cells that showed cross-reactivity with at least one among STRO-1, CD146, and CD44 antibodies, mainly in the paravascular region of human periodontal ligament (Chen et al., 2006). It remains elusive whether BMCs observed in our study were the same as the MSCs/progenitor cells residing in periodontal tissue. It is of interest to clarify the physiological role of BMCs in periodontium and to determine whether BMCs are the origin of MSCs/progenitor cells around blood vessels residing in the periodontal ligament. Further studies are thus necessary.

Next, in order to determine whether BMCs contribute to periodontal wound healing, we created periodontal defects in the bone marrow chimeric mice. Zhou et al. have reported engraftment and differentiation of BMCs to periodontal tissue-forming cells when periodontal defects were treated by regenerative procedure, a grafting of ceramic bovine bone (Zhou et al., 2011). In contrast, Ohta et al. have reported that bone marrow-derived MSCs were not detected in repairing periodontal ligament by a single staining of either STRO-1 or CD44 (Ohta et al., 2008). Our result clearly demonstrated that the number of BMCs around periodontal defects increased as a result of tissue injury in accordance with data reported by Zhou et al. The result that Zhou et al. demonstrated is different from ours in that they investigated the participation of BMCs after periodontal regenerative treatment. In contrast, we investigated whether BMCs are involved in tissue repair without treatment. We observed neither localization of BMCs in alveolar bone 10 weeks post-surgery nor restoration of alveolar bone. It is well known that the tissue debridement abrogates tissue degradation, but does not regain the lost tissue,

implying that our model mimics this clinical situation. It is also possible that periodontal tissue healing in irradiated recipient mice may not represent a physiological process that occurs naturally in non-irradiated mice in response to injury. Thus, it remains to be elucidated whether mobilized BMCs actually contribute to periodontal tissue healing.

Kitaori et al. reported that SDF-1 increased during the repair of bone grafts at both the messenger RNA and protein levels, and that anti-SDF-1 antibody inhibited new bone formation (Kitaori et al., 2009). Moreover, Jones et al. demonstrated that migration of MSCs to bone and bone marrow is CXCR4/SDF-1 dependent in a murine osteogenesis imperfecta model and that SDF-1 up-regulates CXCR4 demonstrating chemotaxis *in vitro* and enhancing engraftment *in vivo* (Jones et al., 2012). It has been reported that inflammation and hypoxia play an important role in regulating the SDF-1/CXCR4 axis. First we confirmed that hypoxia around periodontal defects after periodontal injury (supplemental figure 1). We then checked the expression of SDF-1 in periodontium, and found that SDF-1 expression increased around periodontal defects and in periodontal ligament prior to the recruitment of BMCs (**Figure 4**). It has been reported that SDF-1 levels increase in periodontal disease. (Havens et al., 2008) We confirmed that SDF-1 stimulated the migration of plastic dish-adhered BMCs and that CXCR4-positive BMCs migrated to SDF-1 more promptly than CXCR4-negative BMCs in transwell assay (supplemental figure 2). It has been reported that in BMCs hematopoietic cells, hematopoietic stem cells, MSCs, and endothelial progenitor cells express CXCR4 and that other molecules such as inflammatory factors and cytokines are involved in mobilization of BMCs. (Salem and Thiemermann, 2010) Further experiments are necessary to examine the direct involvement of the SDF-1/CXCR4 axis in recruitment of BMCs *in vivo*. It is also interesting to compare recruitment potential among BMCs.

Adult bone marrow contains several distinct populations of stem cells, such as hematopoietic stem cells, MSCs, endothelial progenitor cells. We observed the number of GFP-positive cells increased in periodontal ligament 5 weeks after injury and about 13% of them are CD45 positive (**Figure 3**). This result suggested that most of cells detected in periodontal ligament were not hematopoietic cells at least at time point we evaluated (5 weeks after injury). It is necessary to directly determine what types of cells are recruited to periodontal defects.

In FACS analyses, we used CD45 (−), TER119 (−), PDGFRα (+), and Sca-1 (+) cells to detect PαS cells, a subset of MSCs in bone marrow. Based on the results of FACS analyses, we demonstrated for the first time that PαS cells in bone marrow decreased after periodontal injury (**Figure 5**). However, it was not clarified whether PαS cells contribute to periodontal tissue repair process. Morikawa et al. reported that PaS-derived clones with multi-differentiation potential are also positive for CD105 and CD90 *in vitro* (Morikawa et al., 2009). It is very important to analyze whether PαS cells that migrated to periodontal defects also express CD90 and CD105.

In summary, our data demonstrate that bone marrow cells are mobilized to periodontal defects. Further experiments are needed to determine cells to be mobilized from bone marrow to periodontal defects and regulatory mechanism involved in this process. Recruitment of BMCs, including a subset of MSCs could be a new target of periodontal treatment.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fcell.2014.00019/abstract

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Cancer-associated mesenchymal stem cells aggravate tumor progression

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Mesenchymal stem cells (MSCs) have both stemness and multi-modulatory activities on other cells, and the immunosuppressive and tumor-promotive mechanisms have been intensively investigated in cancer. The role of MSCs appears to be revealed in tumor aggravation, and targeting MSCs seems to be a promising strategy for treating cancer patients. However, it is still impractical in clinical therapy, since the precise MSCs are poorly understood in the *in vivo* setting. In previous studies, MSCs were obtained from different sources, and were prepared by *ex vivo* expansion for a long term. The inconsistent experimental conditions made the *in vivo* MSCs obscure. To define the MSCs in the host is a priority issue for targeting MSCs in cancer therapy. We recently identified a unique subpopulation of MSCs increasing in mice and human with cancer metastasis. These MSCs are specifically expanded by metastatic tumor cells, and promote tumor progression and dissemination accompanied by immune suppression and dysfunction in the host, more powerfully than normal MSCs growing without interference of cancer. In this review, we summarize current knowledge of the role of MSCs in tumor aggravation, along with our new findings of the bizarre MSCs.

Keywords: mesenchymal stem cell, cancer, metastasis, epithelial-to-mesenchymal transition, immunosuppression

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Introduction

Mesenchymal stem cells (MSCs) have stemness including self-renewability and pluripotency to differentiate into adipocytes, osteocytes, chondrocytes, fibroblasts, pericytes and more, and also have multiple immunoregulatory properties for maintaining immune tolerance (Uccelli et al., 2008; Liu et al., 2009). MSCs suppress immune responses directly by producing immunomodulatory molecules such as IDO, PGE2, TGFβ and nitric oxide, and indirectly via generation and expansion of potent immunosuppressors such as $CD4^+FOXP3^+$ or $CD8^+FOXP3^+$ regulatory T cells (Tregs) and myeloid-derived regulatory cells including dendritic cells (DCregs), monocytes/macrophages (M-MDSCs) and granulocytes (G-MDSCs) (Nauta and Fibbe, 2007; Uccelli et al., 2008; Maggini et al., 2010). Recent studies have demonstrated that MSCs are originally silent, and become immunosuppressive by activation with pro-inflammatory cytokines such as IFNγ, TNFα, and IL-1β, and ligation of TLRs such as TLR2, TLR3, and TLR4 expressed on the MSCs (Burr et al., 2013; English, 2013). This suggests that the functional role of MSCs depends on the components within the microenvironment.

Tumor tissues contain a number of influential factors for recruiting and activating MSCs (Uccelli et al., 2008; Yang et al., 2013). In turn, in the tumor microenvironment, the MSCs modulate

biological properties of tumor cells directly by causing epithelial-to-mesenchymal transition (EMT) followed by induction of tumor metastasis and cancer-initiating stem cells (CSCs), and indirectly via angiogenesis followed by promotion of tumor growth (Uccelli et al., 2008; Yang et al., 2013). These totally lead to tumor heterogeneity responsible for resistance to various treatments. Thus, targeting immunoregulatory and tumor-promotive MSCs seems to be a promising strategy for both attenuating the tumor malignancy, and improving the host immunity against cancer in treating patients.

However, targeting MSCs is still unrealistic in clinical settings, because the *in vivo* MSC profiles remain obscure. The surface molecules expressed in MSCs have been widely investigated, and many studies demonstrated the high expression of CD49a, CD73, CD90, CD105, CD146, CD271, and STRO-1, but not CD11b, CD14, CD19, CD34, CD45, CD79a in human MSCs (Nauta and Fibbe, 2007; Kuhn and Tuan, 2010). However, these molecules are not unique markers specific for MSC phenotype and function. In previous studies, MSCs were mostly obtained from different sources followed by *ex vivo* expansion for a long term. This implies that only a part of MSC subpopulations might be selectively expanded, or transformation in MSCs might generate another type different from the original MSCs in the host. Furthermore, the biological properties of MSCs may be much more modulated under cancer than we have ever known in regenerative research without cancer. These inconsistent experimental conditions impede development of targeting MSCs in cancer therapy. To rigorously define the precise MSCs in cancer patients is a priority issue for the practical application of MSC targeting in the treatment.

We have been exploring novel anticancer therapeutics by focusing on the interplay between neoplastic lesions and host immunity for a while, and have recently provided new insights into the metastatic process (Kudo-Saito, 2013; Kudo-Saito et al., 2013a, 2014). Using murine and human tumor cells with typical features of EMT, high motility and invasivity, following transduction of a cDNA coding for snail family zinc finger 1 (Snail), we found that Snail$^+$ metastatic tumor cells specifically release a large amount of TSP1 (Kudo-Saito et al., 2009), CCL2 (Kudo-Saito et al., 2013b), and FSTL1 (Kudo-Saito, 2013; Kudo-Saito et al., 2013a), all of which can generate immune suppression and dysfunction mediated by immunoregulatory cells including CD4$^+$Foxp3$^+$ Tregs, CD11c$^+$MHC II$^{low/-}$ DCregs and CD45$^-$ALCAM$^+$ MSCs, and functionally impaired CD8^{low} T cells. This pathway totally accelerates cancer metastasis in the host. Among the Snail$^+$ tumor-producing factors, FSTL1, which is a member of the SPARC family (Sylva et al., 2013), is an outstanding molecule playing a dual role in cancer metastasis particularly to the bones. FSTL1 confers the invasive potential and bone tropism on tumor cells, and also generates and expands CD45$^-$ALCAM$^+$ MSCs initially in bone marrow and sequentially all over the body in the host (Kudo-Saito, 2013; Kudo-Saito et al., 2013a). We compared the biological properties between Snail/FSTL1-expanded MSCs (designated "sMSCs") and other MSCs manipulated by none or non-metastatic tumor cells prepared *in vitro* and *in vivo*. The *in vitro* MSCs were CD45$^-$ cells sorted from bone marrow, spleen and peripheral blood of naive mice followed by stimulation with none or culture supernatant of Snail$^-$ or Snail$^+$ tumor cells for 5–7 days. The *in vivo* MSCs were CD45$^-$ cells freshly isolated from those tissues of naive mice or the mice implanted with Snail$^-$ or Snail$^+$ tumor cells (labeled with GFP for elimination). We found that the sMSCs prepared either *in vitro* or *in vivo* specifically produce ANGPT2, CCL2, CCL3, and FSTL1, and most powerfully affect tumor behavior and host immunity by using these molecules (unpublished data except CCL2). ALCAM is expressed only in these MSCs, but not in normal MSCs growing without interference of cancer. These MSCs could be the "activated MSCs" with immunoregulatory properties, since ALCAM is known as an activation marker as well as a MSC marker (Weidle et al., 2010). We validated these results observed in the murine system, using human system with human tumor cells and PBMCs. In addition, we immunohistochemically analyzed tumor tissues of advanced breast cancer patients, and found that accumulation of ALCAM$^+$ cells (possibly including the sMSCs) significantly correlates with FSTL1 expression level in tumor portions, but not in the adjacent normal counterparts. This points to the *in vivo* existence of a causal molecular connection between FSTL1 and ALCAM within the tumor microenvironment in patients.

In another study focusing on human endogenous retrovirus antigen H (HERV-H) that is frequently and highly expressed in metastatic tumor cells, we also found that CD45$^-$CD271$^+$ MSCs are specifically recruited by CCL19 released from HERV-H$^+$ tumor cells in the microenvironment (Kudo-Saito et al., 2014). These MSCs are expanded by a 17-mer peptide that is encoded in the immunosuppressive domain of the HERV-H envelope protein. HERV-H was also found to upstreamly regulate Snail and Twist expressions in the metastatic tumor cells. In the immunohistochemical analysis, the significant correlation between HERV-H and CCL19 expressions and accumulation of CD271$^+$ cells (possibly including the HERV-H-induced MSCs) was observed in tumor tissues of colon cancer patients. This also suggests the clinical relevance of the experimental findings. Later, we additionally found a high similarity of the phenotype and function between the sMSCs and the HERV-H-induced MSCs. Both ALCAM and CD271 are the functional molecules required for cell proliferation and sphere formation of these MSCs, and the soluble factors released by the sMSCs are also upregulated in the HERV-H-induced MSCs (unpublished data). This suggests that the CD45$^-$ALCAM$^+$CD271$^+$ MSCs (also designated "sMSCs" below) could be the cancer-activated MSCs *in vivo*, particularly generated and expanded in the presence of cancer metastasis in patients. The sMSCs would be a promising target for breaking through the difficulty in treating patients with cancer metastasis and impaired immunity. Here, we summarize the current knowledge of the diversified functional role of the bizarre sMSCs (**Figure 1**) including normal MSCs in tumor aggravation.

MSCs Promote Tumor Growth

Because of the high migratory property, MSCs promptly migrate into tumor tissues in response to the inflammatory molecules including chemokines such as CCL5 (Karnoub et al., 2007; Luo et al., 2014) and CXCL16 (Jung et al., 2013). MSCs were

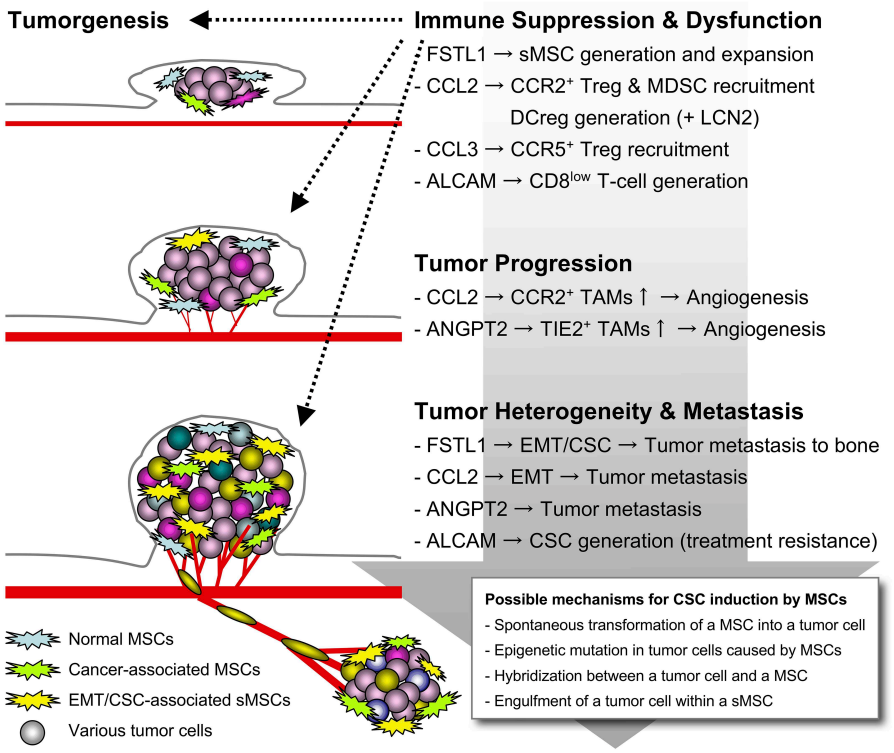

FIGURE 1 | Tumor aggravation caused by the sMSCs. When EMT happens in tumor cells, the Snail^{+} metastatic tumor cells generate and expand multifunctional sMSCs that highly release FSTL1, CCL2, CCL3, and ANGPT2 for potentiating tumor aggravation. The sMSCs promote tumor progression and metastasis by building tumor heterogeneity within the microenvironment, and induce immune suppression and dysfunction for further accelerating the tumor survival and escape.

considered to contribute to tumor progression by causing angiogenesis essential for tumor survival and growth after differentiation into the cancer-associated cells such as fibroblasts (CAFs), pericytes and endothelial cells (Bergfeld and Declerck, 2010). However, the role of the MSC itself begins to be recognized in tumor progression. One paper has demonstrated that CD44 expressed in MSCs is a functional molecule crucial for the migratory and angiogenic properties of the differentiated CAFs (Spaeth et al., 2013). This suggests that the functional role of fibroblasts depend on the original MSCs. In our study, a number of angiogenic factors were significantly upregulated in the sMSCs, and also in the MSCs manipulated by non-metastatic tumor cells as compared to normal MSCs. This seems to be the cancer-associated features. Particularly, ANGPT2 and CCL2 were specifically released by the sMSCs leading to the much more aggressive tumor growth, although no superior production of other potent angiogenic molecules like VEGFs was seen in the sMSCs compared to other MSCs (unpublished data). When the sMSCs were transfected with siRNAs specific for angpt2 or ccl2 before coinjection with tumor cells in mice, the sMSC-induced tumor growth accompanied by increase of F4/80^{+}TIE2^{+} tumor-associated macrophages (TAMs) or CD11b^{+}Gr1^{+} MDSCs in the tumor tissues was significantly inhibited. This suggests that the sMSC-derived ANGPT2 and CCL2 play a key role in the sMSC-induced tumor growth. ANGPT2, which binds to TEK/TIE2, is the antagonist of a potent angiogenic molecule ANGPT1 (Fagiani and Christofori, 2013). However, as well as CCL2, ANGPT2 has been demonstrated to promote tumor growth indirectly via increase of TIE2^{+} TAM-like macrophages in the tumor microenvironment (De Palma and Naldini, 2011). FSTL1 was reported to significantly increase in CAFs within stroma of colon cancer tissues (Torres et al., 2013). However, FSTL1 was a small impact on tumor growth in our study.

Some studies have reported no MSC efficacy on tumor growth (Bergfeld and Declerck, 2010). One possible reason for the paradoxical effects may be the experimental conditions such as MSC sources and *in vitro* culture term. Another possible reason may be the distribution site and the amount of the MSCs *in vivo*. In our experiments, the sMSC effect on tumor growth depended on the route of injection of MSCs into mice. Subcutaneous tumor growth was much more aggressively promoted by subcutaneous (s.c.) coinjection with the sMSCs as compared to the case of intravenous (i.v.) injection, by which only a small and limited amount of the sMSCs spontaneously migrated into the tumor tissues (Kudo-Saito et al., 2013a).

MSCs Promote Tumor Metastasis

Recent studies begin to uncover the roles of MSCs in facilitating tumor metastasis. CCL5 is one of the chemokines produced

from both tumor cells and MSCs in the tumor tissues of breast cancer (Karnoub et al., 2007) and prostate cancer (Luo et al., 2014). Tumor-derived CCL5 recruits MSCs to the microenvironment, and the MSCs also release CCL5 for inducing EMT in the tumor cells. Possibly because the major source of MSCs is bone marrow, the relationship between MSCs and bone metastasis has been intensively investigated (Bergfeld and Declerck, 2010). Bone metastasis is frequently seen in patients, particularly with breast cancers and prostate cancers, giving a high risk that deteriorates the quality of life of patients leading to poor prognosis (Weilbaecher et al., 2011). Previous studies demonstrated that tumor cells migrate into bone marrow in response to various chemokines and cytokines such as CXCL12, CCL2, and IL6, which are released by MSCs (Bergfeld and Declerck, 2010). However, little is known about the molecular mechanisms how MSCs direct tumor cells to the bone.

In our study, we did not observed the significant production of CCL5 and CXCL12 in the sMSCs. Instead, ANGPT2, CCL2, and FSTL1 are significantly upregulated in the sMSCs compared to other MSCs (Kudo-Saito et al., 2013a). The crucial roles of ANGPT2 (Minami et al., 2013; Rigamonti and De Palma, 2013) and CCL2 (Lu et al., 2009; Tang and Tsai, 2012) have been already established in the mechanism of cancer metastasis. We also demonstrated the significance of CCL2 in EMT-associated cancer metastasis (Kudo-Saito et al., 2013b). Later, we additionally found that FSTL1 plays a key role in bone metastasis. FSTL1 can confer bone polarity on tumor cells by inducing the bone metastasis-associated molecular expression such as CCR2, CXCR4, and RANKL (Kudo-Saito et al., 2013a). Cancer metastasis is amplified by CCL2 and FSTL1, which are abundantly produced by both the sMSCs and the metastatic tumor cells within the tumor microenvironment. Interestingly, in addition to the soluble factors, cell-cell contact between tumor cells and the sMSCs is required for tumor metastasis, particularly to bone marrow, where is a niche for maintaining CSCs (Kudo-Saito et al., 2013a). This is also involved in the mechanism of the sMSC-created tumor heterogeneity as described below.

MSCs Create Tumor Heterogeneity and Complexity

It is increasingly being recognized that every tumor cell has a different profile in gene expression and cellular function even if the source (tumor tissue and patient) is just same, and the clonal evolution in a tumor cell builds tumor heterogeneity and complexity governing resistance to the treatments leading to poor outcome in patients (Junttila and De Sauvage, 2013). Although the extracellular matrix is one of the most influential factors for altering tumor properties (Faurobert et al., 2015), MSCs have been also demonstrated to play a key role in generating CSCs with a high metastaticity, dormancy and chemoresistance. In our study, we also observed that tumor cells transform into CSC-like tumor cells following *in vitro* coculture or *in vivo* coinjection with the sMSCs (Kudo-Saito et al., 2013a). This is partly mediated by cell-cell interaction through the ALCAM-ALCAM homophilic binding after ALCAM clustering on the cell surface, which is crucial for proliferation and colonization of CSCs (Weidle et al., 2010) and the sMSCs (Kudo-Saito et al., 2013a). Interestingly, Roodhart et al. reported platinum-based chemotherapy activates MSCs to produce polyunsaturated fatty acids, which confer chemoresistance on tumor cells (Roodhart et al., 2011). This gives a warning in practical settings of treating cancer patients with chemotherapeutics.

In addition to such epigenetic mutations in tumor cells, spontaneous hybridization between tumor cells and MSCs has been recently demonstrated as a non-mutational mechanism in breast cancer (Rappa et al., 2012) and lung cancer (Xu et al., 2014). The tumor/MSC-fused cells have CSC-like properties such as high tumorigenicity and metastaticity, maintaining both profiles of tumor cells and MSCs. In our study, when tumor cells were cocultured with the sMSCs in phagocytosis assay, a few tumor cells were engulfed by the sMSCs, but not fused together, and the tumor cells taken were kept inside of the sMSCs alive without cell death or digestion for a long term (unpublished data). This may be another mechanism that tumor cells lurk with the help of MSCs in the host. To analyze the phenotype and behavior of these tumor-holding MSCs may contribute to further understanding of the mechanism involved in tumor survival and escape. Interestingly, it has been also demonstrated that MSCs transform into tumor-like cells with high proliferative ability following spontaneous mutations (Miura et al., 2006; Tolar et al., 2007; Rosland et al., 2009; Mohseny and Hogendoorn, 2011). This seems to be the fundamental mechanism underlying the emergence of the cancer stem cells originated from non-cancerous cells in the host.

MSCs Protect Tumor Cells from Immune Attack

MSCs cause immune suppression and dysfunction, which totally support all steps of tumor progression (Nauta and Fibbe, 2007; Uccelli et al., 2008). Such immunoregulatory MSCs have been interest in the therapy to properly induce and activate anti-tumor immune responses in cancer patients, since impaired immunity has been a critical issue in the treatment (Uccelli et al., 2008; Yang et al., 2013). Recent studies have demonstrated that MSCs become immunosuppressive following activation through inflammatory signals (Burr et al., 2013; English, 2013). A tumor tissue, where is a milieu filled with abundant pro-inflammatory molecules produced by tumor cells and a variety of the infiltrating cells, could be the best educational place for MSCs to acquire the distinctive competence to support tumor cells. However, how to distinguish between the cancer-activated MSCs and normal healthy MSCs remains to be elucidated.

In our study, we identified CCL2, CCL3, and FSTL1 as the prominent immunomodulatory molecules upregulated only in the sMSCs. The sMSCs generate functionally impaired $CD8^{low}$ T cells through cell-cell contact with ALCAM expressed on the sMSCs (Kudo-Saito et al., 2013a), in addition to regular Tregs as reported elsewhere (Nauta and Fibbe, 2007; Uccelli et al., 2008; Maggini et al., 2010). In the previous studies showing Treg or $CD8^{low}$ T-cell induction by MSCs, CD8 reduction in the $Foxp3^{+}$ T cells (Prevosto et al., 2007), or Foxp3 expression in the $CD8^{low}$ T cells (Giuliani et al., 2011) were not analyzed. The sMSC-derived FSTL1 might amplify such abnormal

immunity by promoting the sMSC expansion in an autocrine manner as well as a paracrine manner by the metastatic tumor-derived FSTL1. CCL2 is also responsible for immunosuppression. In our study, CCL2 orchestrates CCR2-expressing immunomodulatory cells including Tregs, MDSCs and TAMs in the tumor microenvironment, and generates DCregs partly in collaboration with LCN2 that is released from the CCL2-stimulated tumor cells (Kudo-Saito et al., 2013b). Tregs within tumor microenvironment have been demonstrated to highly express a CCL3 receptor, CCR5 (Tan et al., 2009; Schlecker et al., 2012). The sMSC-derived CCL3 may also help to promptly create a beneficial environment for immunoevasion of tumor cells by recruiting the cancer-associated $CCR5^{+}$ Tregs, although not yet investigated in our study.

Concluding Remarks

The significant advances in profiling MSCs provided abundant knowledge about MSCs, and revealed the central role of MSCs in tumor development and progression. The emerging evidences show that MSCs stand out as a promising therapeutic target for treating cancer and the consequent impaired immunity in patients. Nevertheless, we have understood little about the MSCs interacting with tumor cells in the *in vivo* setting. Our sMSC study is gradually unmasking the characteristics of the cancer-activated MSCs that can sharply build heterogeneic and complexed microenvironment beneficial for tumor survival and escape in the host. However, further studies are still needed to achieve translation of the findings to the clinical therapy.

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