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DEVELOPMENT AT A GLANCE

Skeletal stem cells

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ABSTRACT

Skeletal stem cells (SSCs) reside in the postnatal bone marrow and give rise to cartilage, bone, hematopoiesis-supportive stroma and marrow adipocytes in defined *in vivo* assays. These lineages emerge in a specific sequence during embryonic development and post natal growth, and together comprise a continuous anatomical system, the bone-bone marrow organ. SSCs conjoin skeletal and hematopoietic physiology, and are a tool for understanding and ameliorating skeletal and hematopoietic disorders. Here and in the accompanying poster, we concisely discuss the biology of SSCs in the context of the development and postnatal physiology of skeletal lineages, to which their use in medicine must remain anchored.

KEY WORDS: Bone marrow stromal cell, Bone, Cartilage, Hematopoiesis, *In vivo* transplantation, Skeletal stem cells

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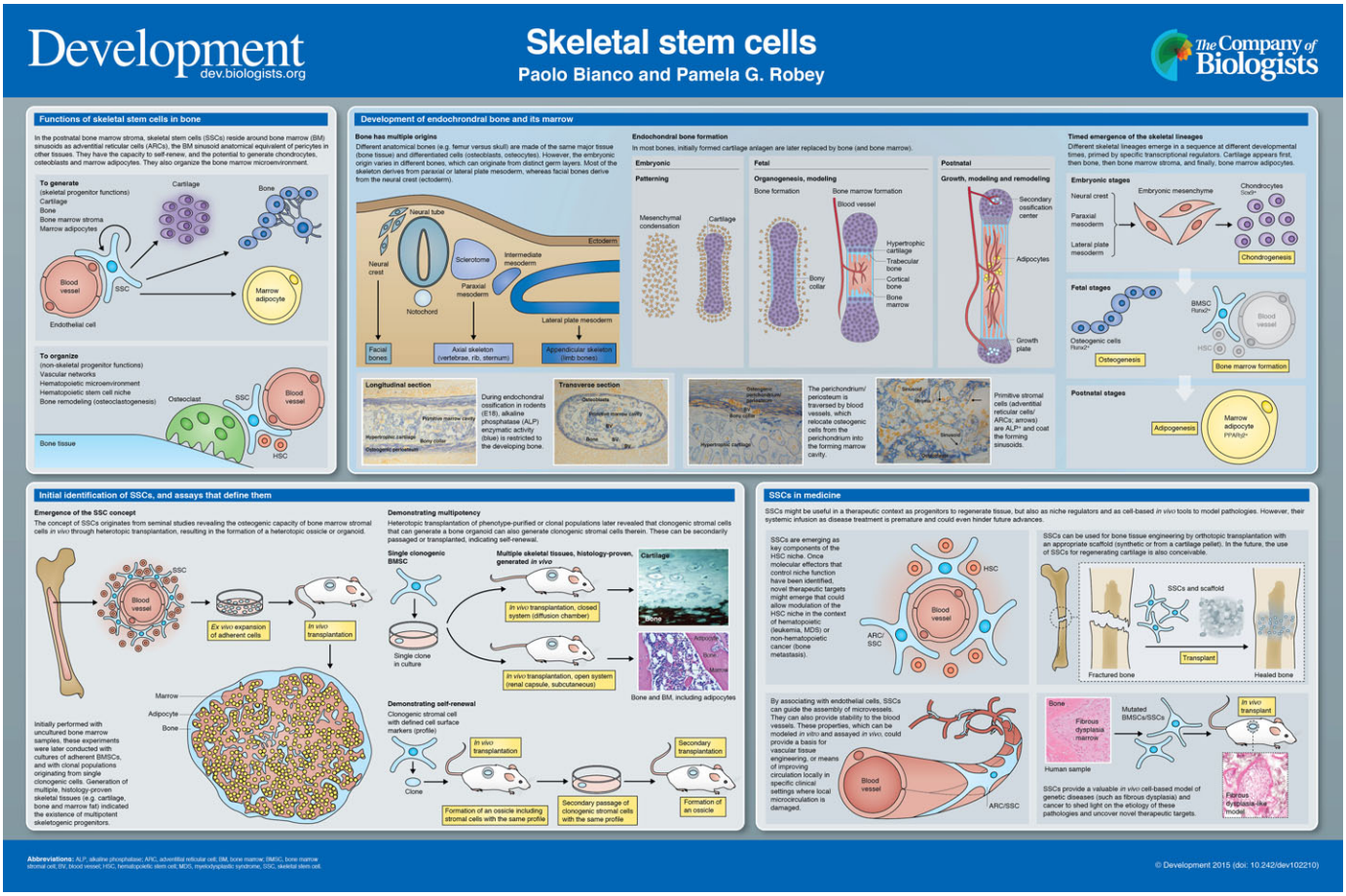
Introduction

Bone marrow (BM) stroma includes self-renewing, multipotent progenitors for skeletal lineages (cartilage, bone, marrow adipocytes, fibroblasts). These progenitors (skeletal stem cells, SSCs) secure a reservoir of bone-forming cells for bone growth during development, bone modeling (sculpting of bone shape) and bone remodeling (life-long bone turnover); they generate adipocytes during growth and during BM remodeling; and under certain circumstances, they form cartilage. BM stromal cells (BMSCs), including SSCs, also shape and regulate the local microvascular network, regulate differentiation of osteoclasts (bone-resorbing cells), and establish and maintain the hematopoietic microenvironment (HME) necessary for growth and blood cell maturation. In addition, they might be essential for retaining long-term self-renewing hematopoietic stem cells (HSCs) (niche function) [reviewed by Bianco et al. (2013, 2008)]. SSCs, which function as skeletal progenitors and as organizers and regulators of the local BM microenvironment, physically exist as perivascular cells (adventitial reticular cells, ARCs) residing at the outer side of the endothelial lining of BM sinusoids, with a distinct phenotype and reticular morphology (Sacchetti et al., 2007; Bianco et al., 2013). Current evidence ascribes both stem cell and organizer



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functions to this specific cell subset, while leaving room for further refinement of potential subpopulations therein.

Here and in the accompanying poster, we summarize the emergence of skeletal lineages throughout embryonic development and postnatal growth and adaptation, as a frame necessary and suitable for understanding the biology of postnatal SSCs. We also briefly consider how the inherent properties of SSCs, sculpted through development, can guide their proper use in medicine, both for understanding and for treating diseases.

Origin of the concept of SSCs

The SSC concept originates from seminal studies whereby heterotopic transplants of intact postnatal BM specimens (devoid of bone) or total BM cell suspensions were found to form an ectopic 'ossicle', comprising multiple skeletal tissues and mimicking the architecture of the bone-bone marrow organ (Friedenstein et al., 1966; Tavassoli and Crosby, 1968). Subsequent work progressively ascribed this function to a specific cell population. Generation of an ossicle was first assigned to adherent (non-hematopoietic) BMSCs; then, to a clonogenic subset thereof, capable of density-insensitive growth (a progenitor), and, more precisely, to a multipotent clonogenic progenitor, giving rise to cartilage, bone and adipocytes, *in vivo* (Owen and Friedenstein, 1988). Finally, self-renewal and anatomical and phenotypic identity of clonogenic, multipotent progenitors (i.e. bona fide SSCs) were recognized (Méndez-Ferrer et al., 2010; Sacchetti et al., 2007; Zhou et al., 2014).

Skeletal, not 'mesenchymal', stem cells

Clarifying the relationship between SSCs and 'mesenchymal stem cells' is not just a matter of semantics. It is essential to elucidate the link between terms, concepts and biological objects, a link often blurred in recent years in the academic and lay usage of terminology. The term 'mesenchymal stem cells' (MSCs), currently used to denote cultures of fibroblastic cells from virtually every tissue or organ (da Silva Meirelles et al., 2006), was in fact first introduced to refer to cultures of BMSCs (Caplan, 1991; Pittenger et al., 1999). The concept of 'MSCs' as non-HSCs in BM was borrowed from the previously formulated hypothesis of an SSC, but was extended to non-skeletal tissues. To date, evidence from rigorous assays indicates that BMSCs include stem cells for skeletal tissues, and skeletal tissues only. Furthermore, such stem cells can be assayed in BM, and BM only. All the extra-skeletal tissues and organs in which 'MSCs' are said to exist (e.g. muscle, placenta, adipose tissue) are developmentally distinct from skeletal lineages, do not contribute to skeletal development or postnatal physiology, do not display skeletogenic properties assayable *in vivo* and are not generated by skeletal progenitors found in the BM. Cells that can be induced to undergo osteogenesis by BMP signaling [including ligand-independent signaling from constitutively active receptors, as in skeletal muscle in the condition fibrodysplasia ossificans progressiva (Medici et al., 2010; Shore et al., 2006)] do exist in other mesoderm derivatives. However, these 'inducible' progenitors must be considered distinct from 'determined' progenitors found in BM stroma, which natively express the osteogenic master gene, runt-related transcription factor 2 (Runx2), and require no reprogramming by exogenous BMPs to generate bone. Additionally, there is no evidence that a stem cell with the characteristics of embryonic mesenchyme, or with multipotency beyond the skeletal lineages, exists in postnatal BM or in the postnatal organism. We therefore believe that the term 'MSC' is a misnomer and should be abandoned.

Skeletal lineages

The ability to generate cartilage, bone, stroma and marrow adipocytes characterizes SSCs. These are all *skeletal lineages*: they are anatomically part of the skeleton. These lineages are related to each other in specific developmental processes and are linked to an assayable postnatal progenitor – the SSC. Cartilage, bone, stroma and marrow adipocytes, like individual bones, develop at different times (with cartilage forming first and adipocytes last) and from multiple embryonic lineages. Facial bones derive from neural crest (ectoderm), whereas the rest of the skeleton derives from paraxial or lateral plate mesoderm (Olsen et al., 2000). Thus, two distinct germ layers and multiple specifications of mesoderm give rise during development to the same range of tissues that are generated by postnatal SSCs. Regardless of developmental origin, BM stroma is found in all bones except auditory ossicles, and studies to date have not revealed significant divergence in the properties of BMSCs from different bones. During organogenesis, cartilage, bone, stroma and marrow adipocytes are generated through a sequence of different fate choices that recall binary choices at different developmental stages – with the early lineages forming exclusively at embryonic times, whereas genesis of later lineages extends into fetal, postnatal and adult life – as discussed further below.

Cartilage and bone

Individual skeletal segments form through two distinct processes, referred to as membranous and endochondral ossification. In the latter, which applies to the vast majority of bones, a cartilage anlage is replaced by bone and BM from within. In the former, a cartilage anlage does not form, or is replaced by bone through a different process, as briefly discussed below. The earliest stage of endochondral ossification during embryonic development is the appearance of mesenchymal condensations. Subsequently, chondrogenesis generates cartilage, which forms anlagen for endochondral bones (Hall, 2005; Kronenberg, 2003). In each condensation, central cells transition to cartilage and then mature further into hypertrophic cartilage, later replaced by bone and BM. The most peripheral mesenchymal cells form an envelope, from which the perichondrium (a layer of connective tissue) and articular soft tissues develop (Hall, 2005). The primitive perichondrium gives rise to both chondrocytes and osteogenic cells, and the two lineages are spatially overlapping at specific sites (Ranvier's grooves) (Hall, 2005). As ossification centers and growth plates are established, peripheral ('borderline') and hypertrophic chondrocytes (HCs) appear to contribute subsets of bone cells (Bianco et al., 1998a; Riminucci et al., 1998; Yang et al., 2014).

In most membranous bones, chondrogenesis is abortive, reflected only by transient expression of type II collagen (Nah et al., 2000). However, a latent chondrogenic phase in membranous ossification can be revealed under specific experimental conditions (Jacenko and Tuan, 1986). Moreover, true cartilage anlagen do form in the development of certain membranous bones (e.g. parietal bones), only to be replaced by bone via a unique process: the anlagen regress by MT1-MMP-driven matrix degradation and apoptosis of resident chondrocytes, while the definitive bone forms outside (rather than inside, as in endochondral ossification) of the regressing cartilage (Holmbeck et al., 1999, 2003).

Bone and BM stroma

Bone tissue forms before a BM cavity is established: bone cells therefore appear before BM osteoprogenitors. Proliferating progenitors of bone-forming cells occupy the outermost part of the perichondrium/periosteum, generating osteoblasts that deposit the 'bony collar' during early ossification. The osteogenic perichondrium

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includes the presumptive BM stromal osteoprogenitors (Arai et al., 2002; Bianco et al., 1993), as confirmed by lineage-tracing studies (Maes et al., 2010). As bone-resorbing osteoclasts perforate the bony collar and underlying HCs, blood vessels from the osteogenic perichondrium/periosteum invade the forming marrow cavity. Periosteal osteoprogenitors, recruited to a perivascular position, relocate to the developing BM (Arai et al., 2002; Bianco et al., 1993; Streeter, 1949). The primitive, pre-hematopoietic BM is formed of large-caliber sinusoid-type blood vessels in an atmosphere of osteogenic cells, some of which reside at the abluminal surface of blood vessels (Bianco et al., 1993). Blood-borne hematopoietic progenitors then seed this environment (Bianco et al., 1999). Throughout prenatal and postnatal bone growth, the BM stroma found at the metaphyses of long bones retains active proliferation; at the same site, angiogenesis fuels growth of the sinusoidal network, and blood-borne hematopoietic progenitors might continue replenishing the locally growing marrow (Wang et al., 2013). Growth of BM stroma at these sites is regulated by parathyroid hormone (PTH) and PTH-related protein (PTHrP), known players in bone development and postnatal physiology. As bones elongate, site-specific downregulation of the PTH1 receptor (PTH1R) might be necessary for abating local ossification, thus making space for steady-state hematopoiesis (Kuznetsov et al., 2004).

BM stroma and adipocytes

Marrow adipocytes develop postnatally from alkaline phosphatase (ALP)-expressing BMSCs located at the outer aspect of sinusoids (anatomically defined as ARCs) (Bianco et al., 1988). ARCs form during fetal development, when committed osteogenic cells associate with blood vessels; these can be isolated as SSCs at postnatal stages (Bianco, 2011). ARCs share their anatomical location (directly beneath the endothelial layer) with cells called pericytes in other tissues, although BM sinusoids are not the same as capillaries in other tissues. ARCs also express markers otherwise expressed in pericytes of other tissues (Bianco, 2014; Sacchetti et al., 2007). Specific regions of the skeleton (apophyses of long bones, short bones, facial bones in humans; tail vertebrae and short bones in mice) are entirely filled with adipocytes at an early age (yellow marrow); the rest of the skeleton is initially filled with hematopoietically active (red) marrow with few or no adipocytes; the number of adipocytes at these sites increases progressively during skeletal growth and aging, but is also modulated by hematopoietic homeostatic needs (Bianco and Riminucci, 1998). Marrow adipocytes are metabolically distinct from extramedullary fat; they contribute to the regulation of blood flow within BM, and, in this way, to hematopoietic activity (Bianco, 2011). They might represent negative regulators of the HSC niche (Naveiras et al., 2009), as much as SSCs/ARCs appear to be a positive regulator. In postnatal BM, a reciprocal relationship (balance) exists between osteogenesis and adipogenesis (Abdallah and Kassem, 2012; Beresford et al., 1992), which is relevant to studies on osteoporosis and bone aging (Justesen et al., 2001). The direct contribution of postnatal skeletal progenitors to remodeling of bone and BM is in need of more intensive studies (Kassem and Marie, 2011).

Regulation of lineage commitment

Developmental commitment to chondrogenesis, osteogenesis and adipogenesis is regulated by three master transcription factors: sex-determining region Y-box 9 (Sox9; chondrogenic) (Bi et al., 1999), Runx2 (osteogenic) (Komori et al., 1997) and peroxisome proliferator-activated receptor γ2 (PPARγ2; adipogenic) (Muruganandan et al., 2009). The levels of these factors are controlled by members of the transforming growth factor β/bone morphogenetic protein (TGFβ/BMP) superfamily, Wnts (wingless type MMTV integration site) and hedgehogs, with extensive crosstalk [reviewed by Cook and Genever (2013)]. In osteogenic commitment, BMP acts via Msh homeobox/distal-less-related (Msx/Dlx) homeoproteins to increase Runx2 expression, which subsequently increases osterix expression. BMPs also play a role in chondrogenesis by upregulating Sox9. In addition, sonic hedgehog (SHH) signaling increases Nkx3.2, which reduces Runx2, allowing for cartilage induction. Wnt signaling prevents cartilage induction, but controls progression of cartilage hypertrophy along with indian hedgehog (IHH) and PTHrP [reviewed by Cook and Genever (2013)]. Adipogenesis is stimulated by hormonal upregulation of CCAAT enhancer-binding protein (C/EBP) β and δ, which in turn induces PPARγ2. PPARγ2 upregulates C/EBPα, which maintains PPARγ2 expression via a positive-feedback loop. Of note, Wnt signaling promotes osteogenesis by stimulating Runx2 and by inhibiting C/EBPα, thereby inhibiting adipogenesis [reviewed by Cook and Genever (2013)]. In addition, TAZ (transcriptional coactivator with PDZ-binding motif) co-activates Runx2-dependent gene transcription while repressing PPARγ-dependent gene transcription (Hong et al., 2005).

Generating skeletal progenitors from pluripotent stem cells

Study of pluripotent stem cells (PSCs; including embryonic stem cells, ESCs; and induced pluripotent stem cells, iPSCs) provides a way to dissect out the regulatory circuits governing human bone and marrow development, including the origin of SSCs. Many studies have aimed at differentiating ESCs or iPSCs into skeletal tissues [e.g. Harkness et al. (2011); Mahmood et al. (2010)]; several have shown that putative PSC-generated skeletal progenitors could establish histology-proven bone *in vivo*, but failed to recreate a BM cavity and stroma, and therefore SSCs (Harkness et al., 2011; Hong et al., 2014; Mahmood et al., 2010; Phillips et al., 2014). This finding resonates with the current inability to generate genuine HSCs while generating hematopoietic cells from PSCs (Kaufman, 2009). It is also worth noting that BM stroma and SSCs comprised therein emerge late during bone organogenesis (see above), suggesting that current protocols for generating SSCs from PSCs might fall short of recapitulating the entire process of skeletogenesis, particularly its late events. More generally, the specific characterization of the properties of skeletal progenitors at specific points in developmental time and space stand as an important task ahead.

Isolation and assessment of skeletal stem/progenitor cells

Skeletal progenitors can be isolated based either on surface markers (‘prospectively’) or by establishing clonal adherent cultures. As long as clonogenicity assays remain the mainstay of characterization of cells isolated based on surface markers, and as long as cell culture remains necessary prior to transplantation *in vivo*, isolation by either surface marker or by adherence and clonogenicity yield essentially identical results. Multipotency can only be assayed at the single cell level; i.e. in clones originating from a single colony-forming unit-fibroblast (CFU-F). All analyses of single clones have confirmed a major diversity across clones (growth and differentiation capacity) [e.g. Kuznetsov et al. (1997)], possibly reflecting a hierarchy (Muraglia et al., 2000). Although hugely popular, assessment of immunophenotype *in vitro* and assays of multipotency in non-clonal cultures do not define a culture as a culture of SSCs or of stem cells (Bianco et al.,

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2013), and significantly confound the assessment of stem cell growth, differentiation and self-renewal.

Cell proliferation and colony growth

Regardless of phenotypic homogeneity, all cultures of non-transformed cells are heterogeneous, as their growth kinetics are necessarily asymmetrical: further proliferation, differentiation and senescence of the progeny of a single cell do not occur uniformly, synchronously or at the same rate for all cells in a growing colony. Furthermore, fully symmetrical growth kinetics of a population originating from a single stem cell would expand the stem cell number without generating differentiating cells; or, conversely, would generate differentiating cells without increasing the number of stem cells. As we have no clue about the relative frequency of symmetric versus asymmetric cell divisions in the cultures, the actual content of true stem cells, or progenitors, in a culture cannot be controlled at this time. Thus, referring to any culture of BMSCs as a culture of 'stem cells' or of 'mesenchymal stem cells' (as in a copious amount of literature) remains ungrounded and misleading.

Differentiation

Heterotopic transplantation of non-doctored, non-induced cultures remains the mainstay for assessing the differentiation capacity of SSCs/BMSCs (Bianco et al., 2008). Surrogate *in vitro* assays are highly artificial and prone to artifact. This applies particularly to osteogenic and adipogenic differentiation assays, even if combined with limited analyses of gene expression. Cartilage formation in micromass or pellet cultures is more reliable, as it rests on ultimate histological proof of genuine cartilage. However, like the osteogenic and adipogenic assays, loose interpretation of pellet culture results plagues its use (Robey et al., 2014). Cartilage pellets can in turn be transplanted, to the effect of generating ossicles through a unique developmental sequence (Serafini et al., 2014).

Self-renewal

Self-renewal can only be assessed by proving the generation, *in vivo*, of a cell compartment that is anatomically, phenotypically and functionally equivalent to the one originally explanted, along with differentiated compartments. This requires: (a) definition of the anatomy and phenotype of the isolated population; (b) evidence that the same population is established *in vivo*; and (c) serial passaging and transplantation (Sacchetti et al., 2007). Extensive numbers of population doublings (PDs) do not prove self-renewal of post-natal cells, but simply a high proliferation capacity.

SSCs in medicine

There are four ways in which SSCs might be useful in therapeutic contexts, including patient treatment: (1) as progenitors, for example, of bone cells for bone tissue engineering (Robey, 2011); (2) as non-progenitors, for example, by guiding microvessel assembly and networking, as key components of the HME/niche or as suppliers of factors and cytokines in the context of specific tissue-engineering strategies (Bianco, 2011; Bianco et al., 2013); (3) as modeling tools in stem cell-based *in vivo* models of disease (Bianco et al., 1998b); and (4) as a conceptual tool, by revealing specific pathological changes in tissues as a result of dynamics in stem cell biology. It is important to understand these dynamics for therapeutic purposes, as drug-targetable processes might be operating in the stem or progenitor cells rather than in the differentiated cells (Bianco, 2014; Bianco and Riminucci, 1998). All of these modes of SSC impact in medicine need to be harnessed in specific ways, yet to be devised. A premature, empirical rush to clinical application (e.g. by systemic infusion of cells that are not amenable to systemic infusion) will not provide advances and might even prevent them (Bianco et al., 2013).

Concluding remarks

The skeleton provides a key example of a low-turnover system in which bona fide stem cells have been proven to exist and have been characterized in humans and mice (Méndez-Ferrer et al., 2010; Sacchetti et al., 2007). This was carried out using experimental approaches germane to those that revealed the biology of other stem cells systems (i.e. hematopoietic), such as transplantation and single-cell analysis. Recent developments of classical approaches have been instrumental in identifying the SSCs, and remain crucial to the endeavor of probing their functions and regulation in humans. Very recently, advances towards identifying SSCs in the mouse have been reported (Chan et al., 2015; Worthley et al., 2015). These studies reveal novel facets of the ontogeny of the stromal system. Together with a more thorough appreciation of the specific developmental processes briefly discussed herein, these new data might contribute to a broader understanding of the stromal system in mammals.

SSCs have a unique role: they function at the same time as stem cells and as niche cells, as progenitors and as organizers of neighboring cells and tissues that they do not generate, such as nascent blood vessels and the HME. This singularity might rest on cellular properties and regulatory circuitries still to be elucidated. As central to the physiology and disease of two major systems such as the skeleton and blood, SSCs might provide conceptual and practical advances in medicine, provided their specificities are kept in mind.

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Competing interests

The authors declare no competing or financial interests.

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No Identical "Mesenchymal Stem Cells" at Different Times and Sites: Human Committed Progenitors of Distinct Origin and Differentiation Potential Are Incorporated as Adventitial Cells in Microvessels

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SUMMARY

A widely shared view reads that mesenchymal stem/stromal cells ("MSCs") are ubiquitous in human connective tissues, can be defined by a common in vitro phenotype, share a skeletogenic potential as assessed by in vitro differentiation assays, and coincide with ubiquitous pericytes. Using stringent in vivo differentiation assays and transcriptome analysis, we show that human cell populations from different anatomical sources, regarded as "MSCs" based on these criteria and assumptions, actually differ widely in their transcriptomic signature and in vivo differentiation potential. In contrast, they share the capacity to guide the assembly of functional microvessels in vivo, regardless of their anatomical source, or in situ identity as perivascular or circulating cells. This analysis reveals that muscle pericytes, which are not spontaneously osteochondrogenic as previously claimed, may indeed coincide with an ectopic perivascular subset of committed myogenic cells similar to satellite cells. Cord blood-derived stromal cells, on the other hand, display the unique capacity to form cartilage in vivo spontaneously, in addition to an assayable osteogenic capacity. These data suggest the need to revise current misconceptions on the origin and function of so-called "MSCs," with important applicative implications. The data also support the view that rather than a uniform class of "MSCs," different mesoderm derivatives include distinct classes of tissue-specific committed progenitors, possibly of different developmental origin.

INTRODUCTION

The anatomical identity of mesenchymal stem/stromal cells ("MSCs," the current "jargon"), their phenotype, distribution in different tissues, lineage, physiological functions, and biological properties represent one of the most controversial and confusing areas in stem cell biology. At this time, two quite distinct descriptions of "MSCs" are found in the literature. One, which emanates from ~50 years of widely reproduced experimental work in vivo, sees "MSCs" as the same biological object previously known as cultured bone marrow stromal cells (BMSCs); these cells are unique to bone marrow (BM), and include a subset of physically identifiable clonogenic, multipotent, self-renewing progenitors of skeletal tissues, and skeletal tissues only (Bianco et al., 2013). This progenitor is endowed with the unique capacity to organize the hematopoietic microenvironment and the hematopoietic stem cell niche (Bianco, 2011; Friedenstein et al., 1982). The other view sees "MSCs" as progenitors of multiple tissues beyond the range of skeletal tissues, such as skeletal muscle (Caplan, 1991, 2008; Crisan et al., 2008). The demonstration that "MSCs" are perivascular cells in BM (Sacchetti et al., 2007) was later extrapolated to claim that in virtually all tissues, pericytes (identified as $CD34^-/CD45^-/CD146^+$ cells) would represent "MSCs" (Caplan, 2008; Crisan et al., 2008). Hence, these broadly multipotent progenitors, essentially defined by in vitro assays (Dominici et al., 2006; Pittenger et al., 1999) that are neither specific nor stringent, would be found in multiple tissues well beyond BM (e.g., skeletal muscle, fat, placenta, umbilical cord) (Caplan, 2008; da Silva Meirelles et al., 2006).

Definition of the origin, anatomy, biological properties, and function of so-called "MSCs" has obvious implications, both for understanding their biology and for their use in potential therapies. Notably, assuming that "MSCs" with identical differentiation properties can be isolated from virtually every tissue would imply that multiple tissues are equally suitable cell sources for the regeneration of multiple tissues. On the other hand, the assumption that

"MSCs" are the ex vivo counterpart of pericytes would lend support to the view that a number of non-progenitor functions (Bianco et al., 2013) of "MSCs" (anti-inflammatory, immunomodulatory, trophic), claimed to be of major import for therapy of a number of unrelated disorders (Caplan and Correa, 2011), are traceable to an identifiable and ubiquitous in vivo cell type. Nonetheless, pericytes are only defined by anatomy, and currently no experimental data support the notion that they represent a distinct lineage (Armulik et al., 2011; Diaz-Flores et al., 2009). In addition, their role in tissue injury and repair is pleiotropic and spans multiple distinct processes including inflammation; furthermore, their participation in the repair of tissues (e.g., through the formation of scar tissue) does not necessarily coincide with a regenerative function.

We previously identified a minimal surface phenotype suited not only to enrich the archetypal human "MSCs" in uncultured BM cell suspensions, but also to correlate their ex vivo-assayed clonogenic capacity with their in situ identity and in vivo fate following transplantation (Sacchetti et al., 2007). As applied to the study of BMSCs, this led to identification of "MSCs" as subendothelial, perivascular CD146$^+$ cells on BM sinusoids, and also provided evidence for their self-renewal in vivo, which had long been the missing evidence to support the claim that BMSCs indeed include a subset of bona fide stem cells, rather than multipotent progenitors (Bianco et al., 2013; Sacchetti et al., 2007). Using an identical approach to prospectively isolate "MSCs" from a variety of non-BM tissues, Crisan and co-workers later reported that a ubiquitous population of highly myogenic and skeletogenic CD146$^+$ cells, coinciding with "MSCs," is found in association with microvessels of skeletal muscle and other tissues, lending support to the view of pericytes as a uniform, widely distributed population of cells that can be explanted and cultured as "MSCs" (Caplan, 2008; Caplan and Correa, 2011; Crisan et al., 2008). However, striated muscle and skeletal lineages such as bone, cartilage, and marrow fat diverge early in development, and no common progenitor of bone and muscle is found in prenatal life past the time of sclero-myotome specification in somites (Applebaum and Kalcheim, 2015). The notion of a common postnatal progenitor of bone and muscle, therefore, would be at odds with established tenets in developmental biology (Bianco and Robey, 2015).

We show here that MCAM/CD146-expressing stromal cells from different human tissues diverge radically from their BM counterparts in differentiation potency and transcriptional profile, reflective of their different developmental origin. While BM-derived "MSCs"/pericytes are natively skeletogenic but not myogenic, muscle-derived "MSCs"/pericytes are inherently myogenic but not natively skeletogenic, and appear to represent a subset of cells with functional features of satellite cells, but not their characteristic anatomical location. We further show that prenatal, cord blood-borne "MSCs" in turn exhibit a distinct transcriptional and potency profile, and an inherent cartilage commitment, which diverge markedly from that of postnatal BM-derived "MSCs." Finally we show that, irrespective of the postnatal tissue source of these perivascular cells or from fetal blood, these committed progenitors of mesoderm derivatives can associate with nascent blood vessels (BVs) in vivo and be recruited to a mural cell fate. However, a system of committed and self-renewing progenitors with distinct native potency, and not a uniform, equipotent class of "MSCs" is associated with microvascular walls in postnatal mesoderm-derived tissues as reported previously for bone/marrow (Sacchetti et al., 2007), and as shown herein for muscle. Pericyte recruitment from preexisting local progenitors is a simple developmental process that explains the very existence of such progenitors in postnatal life and their tissue-specific properties.

RESULTS

The Phenotype of "MSCs" In Vitro Does Not Reflect Cell Identity and Function

Stromal cell strains were established from four different tissue sources: BM, skeletal muscle (MU), periosteum (PE), and perinatal cord blood (CB). For all postnatal tissue sources, clonogenic cells were prospectively isolated based on a minimal surface phenotype as previously described for human BMSCs (CD34$^-$/CD45$^-$/CD146$^+$); colonies of CB stromal cells were established as described previously (Kluth et al., 2010; Kogler et al., 2004). Of note, CD146 identified a clonogenic subset in MU (presented below) and PE (data not shown), as it does in BM. Multiclonal strains derived from growth of the originally explanted cells were then expanded under identical basal culture conditions that do not support the growth of endothelial cells or induce differentiation. All resulting cell strains exhibited the canonical in vitro cell-surface markers regarded as characteristic of "MSCs" (Figure 1A).

To determine the specificity and functional significance of the cell-surface phenotype of "MSCs," widely regarded as a defining feature of "MSCs" across tissues, we performed gene-expression profiling using Affimetrix technology. Both unsupervised hierarchical clustering (Figure 1B) and principal component analysis (Figure 1C) revealed that gene-expression profiles of "MSCs" are clearly separated by an "origin" factor, indicating the lack of specificity and sensitivity of the widely used "minimum" surface phenotype. ANOVA-based supervised analysis selected 1,614 class-specific, differentially expressed genes (Table S1) showing a fold difference >3 and a false discovery rate

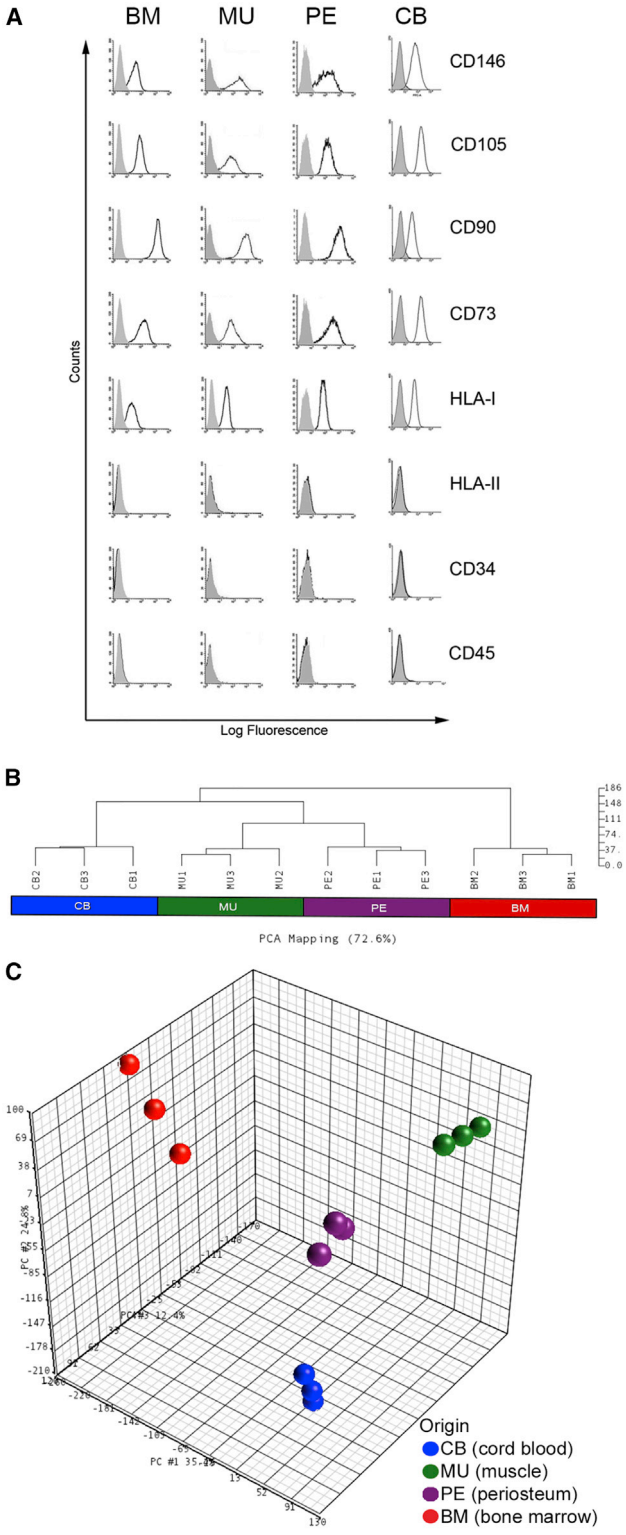

Figure 1. Cell Surface and Transcriptomic Comparison of "MSCs" from Different Tissues

(A) Fluorescence-activated cell sorting (FACS) analysis of multiclonal BM (bone marrow), MU (muscle), PE (periosteum) and CB (cord blood) cells (representative of one from least three independent experiments). All strains express the canonical in vitro phenotype of "MSCs" and CD146 (isotype controls indicated in gray). (B and C) Unsupervised clustering of gene-expression profiling data of CD146$^+$ cells purified from BM, CB, MU, and PE (three independent samples for each cell type). Unsupervised analysis was performed to investigate whether there was evidence for native groupings of samples based on correlations between gene-expression profiles. Results of the hierarchical clustering (B) and the principal component analysis (C) revealed that gene-expression profiles of CD146$^+$ cells are clearly separated by the origin factor. See Supplemental Experimental Procedures for further details of statistical analyses.

q value of <0.05. While perinatal CB cells are characterized by over-representation of genes related to proliferation and cell-cycle regulation (Figures 2A–2C), the postnatal MU cells are characterized by over-representation of tissue-specific genes related to their tissue origin (Figures 2D and 2E), including the myogenic transcription factor, *PAX7* (Figure S1A). Tables S2 and S3 show the first 100 enriched gene sets for CB and MU classes, respectively, while Figures 2A1–2E1 show enrichment plots and heatmaps for selected gene sets. The over-represented gene sets coming from gene set enrichment analysis (GSEA) (Subramanian et al., 2005) support the notion that prospectively purified CB "MSCs" are highly proliferative, since the majority of gene sets enriched in this phenotype are related to proliferation, S phase, RNA and DNA synthesis, or DNA repair. On the other hand, prospectively purified MU "MSCs" are clearly characterized by the over-representation of gene sets specifically related to either muscle development or muscle differentiated function (muscle contraction, muscle development, and energy metabolism). PE and BM expression profiling was analyzed in the same way, but no gene sets were statistically significantly enriched in PE versus CB, BM, and MU, or in BM versus PE, CB, and MU. However, a number of genes enriched in BM and PE cells was identified (Table S4). Furthermore, genes associated with hematopoietic support, a defining feature of BM cells, were over-represented in BM cells compared with CB, MU, and PE cells (Figure S2A).

"MSCs" from Different Sources Have Radically Different Differentiation Properties

BM "MSCs," prospectively sorted as CD34$^-$/CD45$^-$/CD146$^+$ and grown under basal conditions that do not induce differentiation, regularly form bone and establish the hematopoietic microenvironment when transplanted heterotopically using an osteoconductive carrier (Sacchetti et al., 2007) (Figure 3Aa). Cells sorted based on the same phenotype from BM and other tissues, including MU, were later reported to be highly myogenic both in vitro

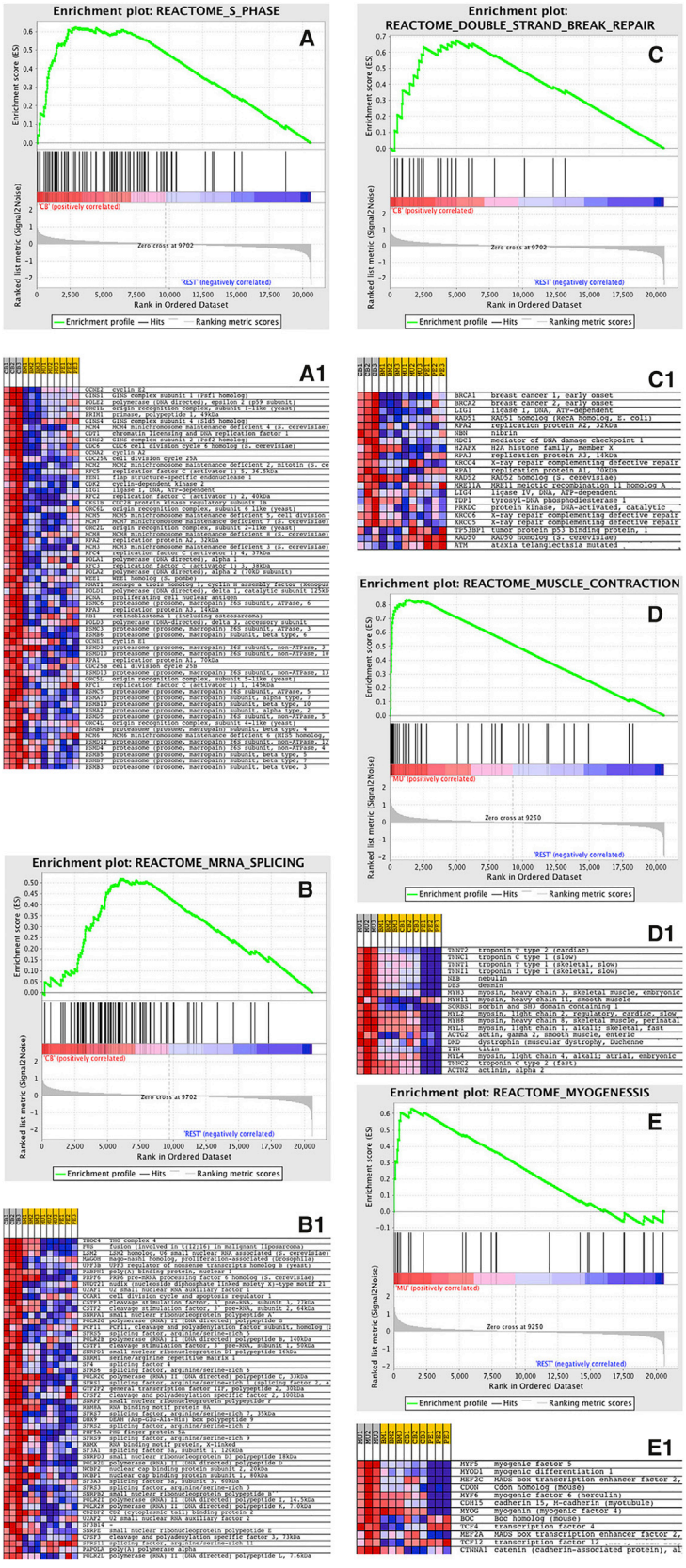

Figure 2. Enrichment Plots and Heat Maps of Selected Gene Sets for Cord Blood- and Muscle-Derived CD146$^+$ Cells

(A–C1) Prenatal CB (cord blood) "MSCs" are characterized by the over-representation of many genes related to proliferation and cell-cycle regulation.

(D and E) Postnatal MU (muscle) "MSCs" are characterized by the over-representation of tissue-specific genes related to their tissue origin, specifically by genes regulating muscle contraction, muscle development, and energy metabolism. The over-represented gene sets from GSEA showed that prenatal CB CD146$^+$ cells are enriched in gene sets related to proliferation S phase, RNA and DNA synthesis, or DNA repair.

For each enriched gene set, the gene expression is also represented as a blue-pink o'gram in (A1), (B1), and (C1) (CB "MSCs"), and (D1) and (E1) (MU "MSCs"). See Supplemental Experimental Procedures for further details of statistical analyses.

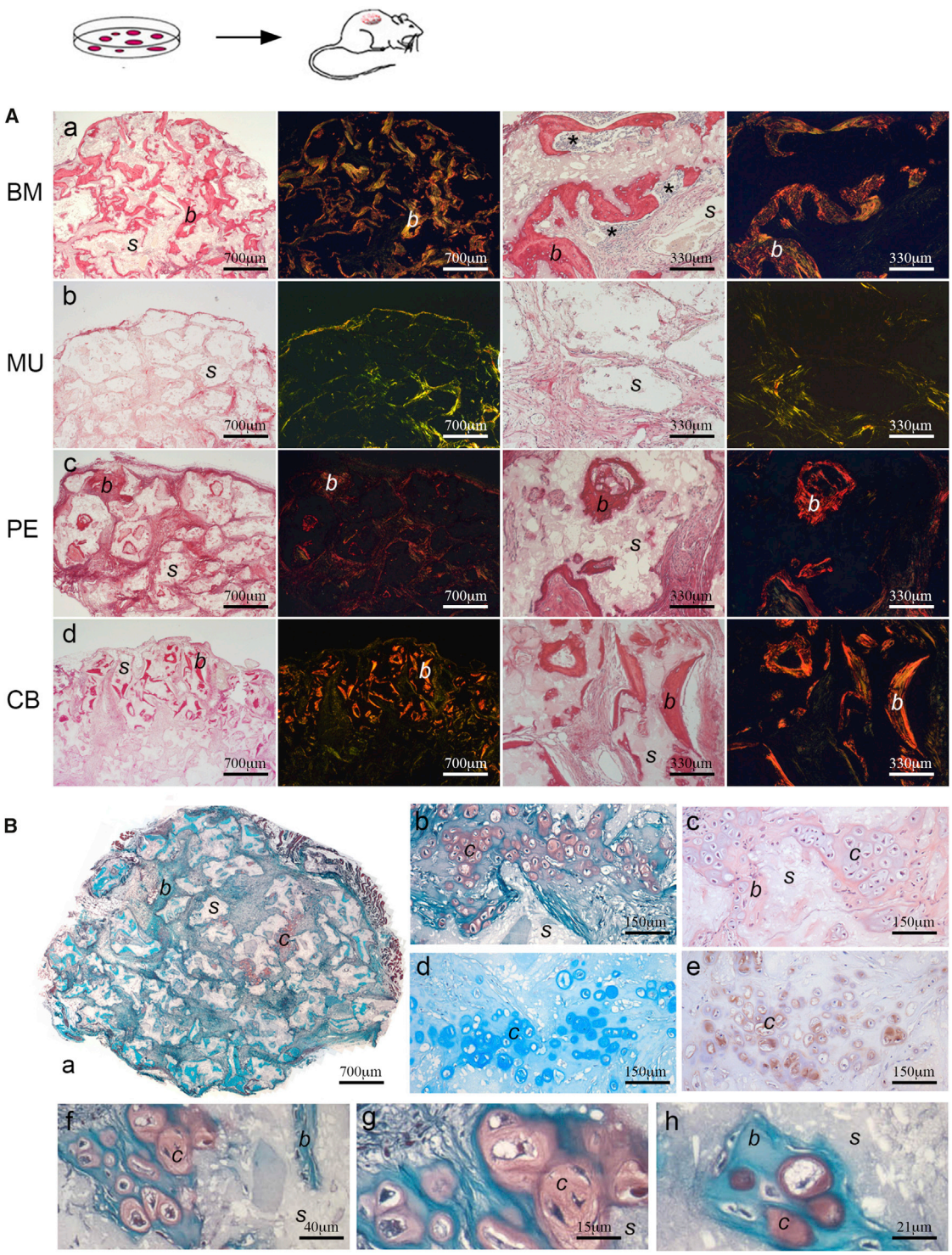

Figure 3. In Vivo Transplantation of CD146$^+$ "MSCs" Derived from Different Tissues

(A) In vivo osteogenic differentiation of BM (bone marrow, a), MU (muscle, b), PE (periosteum, c), and CB (cord blood, d) cells (representative results of one from at least three transplants). Sirius red (columns 1 and 3) stains bone (labeled *b*) intensely due its high collagen content. Polarized light (columns 2 and 4) shows the distribution of Sirius red-stained collagen fibers in bone. When CD146$^+$ "MSC" strains were transplanted, using the same in vivo assay with hydroxyapatite/tricalcium phosphate (HA/TCP) as a scaffold (labeled *s*), osteogenic potential was restricted to BM-, PE-, and CB-derived cells. MU cells regularly failed to form any histologically identifiable bone.

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and in vivo, in addition to sharing the ability to differentiate in culture toward skeletal lineages (Crisan et al., 2008), based on widely used artificial differentiation assays. In vitro, Alizarin red S and von Kossa staining cannot distinguish between dystrophic calcification induced by dead and dying cells versus matrix mineralization, or calcium phosphate precipitates generated by cleavage of β-glycerophosphate (a component of osteogenic medium) by alkaline phosphatase (ALP), which is expressed by many types of stromal cells. In vivo transplantation of MU "MSCs" of identical surface phenotype as BM "MSCs" revealed no spontaneous in vivo osteogenic potential (Figure 3Ab). Likewise, cells established in culture from skin, adipose tissue, and amniotic fluid, all sharing the in vitro phenotype of "MSCs," regularly failed to form any histology-proven bone (Figure S2), whereas PE "MSCs" did form bone in vivo, as previously reported (Sacchetti et al., 2007; Figure 3Ac). Using the same in vivo assay and carrier, CB "MSCs" formed histology-proven bone of donor origin (Figure 3Ad, human Lamin A/C-positive osteocytes, not shown). Surprisingly, they also generated Safranin O^+, Alcian $blue^+$, $COL2^+$ hyaline cartilage intermingled with bone in the same assay (three of three strains from different donors, and one of three single colony-derived strain), but never established a hematopoietic microenvironment (Figures 3Ba–3Bh). This result was unique, as previously we have never seen BM "MSCs" make cartilage in this ceramic-based assay. Failure to generate cartilage under these conditions has been interpreted as a need for a hypoxic environment for chondrogenesis in vivo, which is not provided in an open transplantation system permissive for vascularization. While hypoxia undoubtedly contributes to chondrogenesis (as in fracture callus formation; e.g., Hirao et al., 2006), our results suggest that there are also cell-intrinsic factors at play in CB-derived chondrogenesis based on their more primordial, fetal origin (Bianco and Robey, 2015).

The myogenic capacity of the same cell strains was tested under stringent conditions (i.e., in the absence of exogenous myoblasts in vitro or endogenous myoblasts in vivo [Sherwood et al., 2004], of demethylating agents such as 5-azacytidine [Wakitani et al., 1995]). MU "MSCs" cultured without myoblasts in canonical myogenic conditions (2% horse serum on Matrigel) (Dellavalle et al., 2007) readily and efficiently generated myotubes in culture, whereas no myogenesis was observed under identical conditions with non-MU-derived stromal cells (Figure 4A). With these non-MU "MSCs," myotubes could only be obtained in co-cultures with myoblasts (mouse C2C12 cells). Notably, the vast majority of nuclei incorporated in the newly formed myotubes were murine, indicating that human non-MU "MSCs" contribute only marginally to myogenesis in vitro even in permissive co-culture differentiation assays (data not shown). In cultures of MU $CD146^+$ cells (Figure 4C) highly efficient myogenesis was observed, even in colonies established by single $CD146^+$ cells (Figure 4B), and occurred spontaneously as the colonies became dense (13/32; 41%) as observed with bona fide myoblasts (Figure 4D).

Local transplantation of $CD146^+$ BM, PE, or CB cells into cardiotoxin (CTX)-injured tibialis anterior muscle of SCID/beige (Figure 5A) or SCID/mdx mice (data not shown) (Dellavalle et al., 2007) failed to contribute to regenerating muscle. In contrast, $CD146^+$ MU cells revealed an efficient incorporation of donor nuclei (Figures 5B; Tables S5A and S5B), and expression of human muscle proteins in regenerating myofibers (Figure 5C [CD56], Figure 5D [Dystrophin 2, Spectrin], and Table S5C). Subsequently, enzyme-released cells of the harvested injected TA were used to perform secondary MU colony-forming efficiency (CFE) assays (Supplemental Experimental Procedures; Figure S3). Human cells were isolated based on hCD44, hCD90, and hCD146 expression and, after brief expansion (2 weeks), reanalyzed by fluorescence-activated cell sorting (FACS) for hCD44, hCD90, and hCD146 expression. The human cells were then isolated by MiniMacs (Miltenyi) and were replated in culture at clonal density. All colonies harvested at 2 weeks were uniformly positive for hCD146 and negative for hCD56 and hCD34, demonstrating that $hCD146^+$ pericytes isolated from the injected muscle were the source of the secondary MU colony-forming unit-fibroblasts (CFU-Fs), indicative of self-renewal.

The canonical in vivo muscle regeneration assay relies in large part on fusion between donor cells and host myoblasts, which implies recruitment of the former to a newly forming myotube where they will be reprogrammed by host myogenic factors. Even though CB, PE, and BM cells failed to differentiate under these conditions, a more stringent in vivo myogenesis assay requires the exclusion of such recruiting myoblasts. To this end, we suspended equal

PE and CB "MSCs" formed bone, but never established a hematopoietic microenvironment. BM "MSCs" formed bone and established the hematopoietic microenvironment (asterisks in a, third panel), which is assessed by determining the presence of extravascular blood cells such as megakaryocytes and granulocytes, as seen under high-power microscopy (not shown). Scale bars represent 700 μm.
(B) Clonal strains of CB $CD146^+$ "MSCs" transplanted using HA/TCP as a scaffold (representative results from one of at least three transplants) generated abundant bone and hyaline cartilage (labeled *c*). a, b, f–h: Safranin O (stains chondrocytes and cartilage orange due to their proteoglycan content) and Fast green (stains bone matrix deep green-blue); c: H&E, whereby unlike bone which stains pink with eosin, cartilage stains light blue; d: Alcian blue (stains cartilage matrix); e: anti-type II collagen (an essential cartilage component) immunohistochemistry. Scale bars represent 700 μm (a), 150 μm (b–e), 40 μm (f), 15 μm (g), and 21 μm (h).

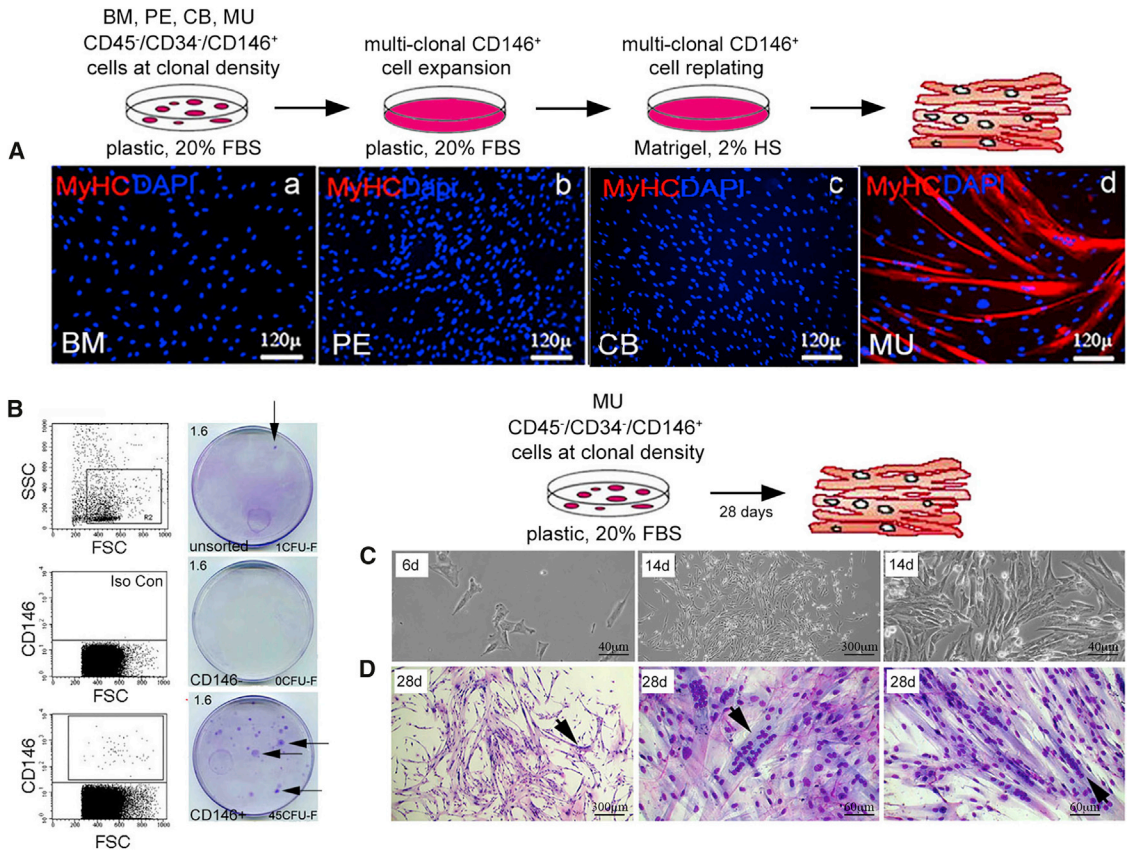


Figure 4. In Vitro Myogenic Assay of CD146$^+$ "MSCs" Derived from Different Tissues

(A) Conventional in vitro differentiation assays were conducted using multiclonal "MSCs" from BM (bone marrow, a), PE (periosteum, b), CB (cord blood, c), and MU (muscle, d) cells, which were first expanded on plastic with 20% FBS, and then replated on Matrigel with 2% horse serum (representative results from one of at least three independent experiments). With MU CD146$^+$ "MSCs," extensive formation of myotubes expressing specific myogenic markers were observed. No myogenic differentiation was observed with any non-MU-derived cell strain. MyHC, myosin heavy chain. Scale bar represents 120 μm.

(B) Freshly isolated MU CD146$^+$ cells were plated at clonal density (1.6 cells/cm^2, indicated in upper left corner). At clonal density, MU CD146$^+$ but not MU CD146$^-$ cells formed discrete fibroblastic colonies (arrows point to colonies, number of colonies formed indicated in lower right corners, Giemsa stain) (representative results from one of least three independent experiments).

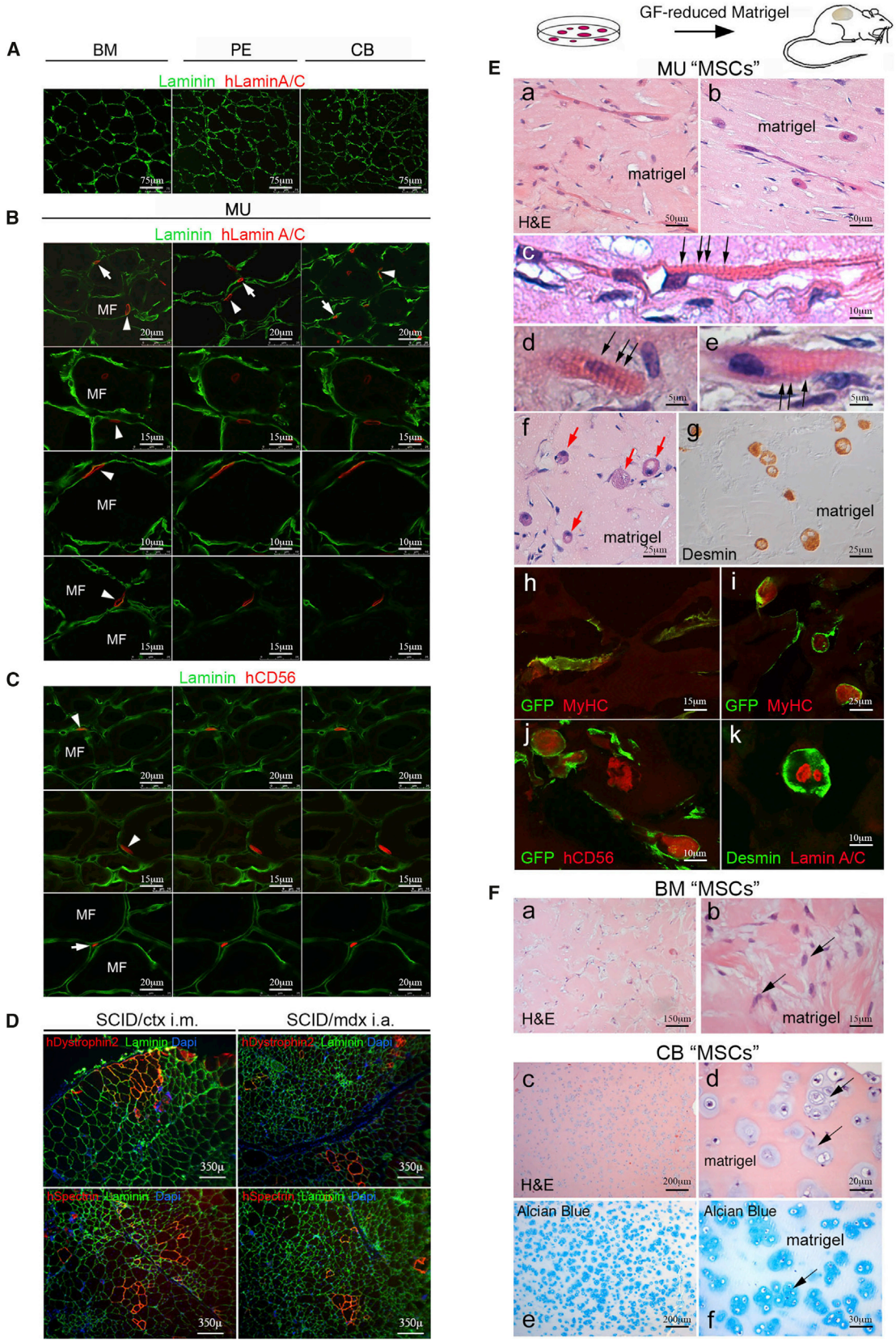
(C) Cell morphology in single colonies generated by CD45$^-$/CD34$^-$/CD146$^+$ muscle-derived clonogenic cells at 6 days (scale bar, 40 μm) and 14 days (scale bars, 300 μm and 40 μm) in culture, in 20% serum, and on plastic (results from one experiment).

(D) Three such colonies of MU cells, one at low power (scale bar, 300 μm) and two at high power (scale bar, 60 μm), at 28 days without passage. With time and without passage, colonies become dense and 13 of 32 (41%) underwent spontaneous differentiation. Multinucleated myotubes are obvious after Giemsa staining (arrows).

numbers of MU (transduced to express a cell-surface GFP label), BM, or CB cells with identical surface phenotype, grown in culture under identical basal conditions that did not induce differentiation, in growth factor-reduced Matrigel, and injected the resulting suspension into the epifascial space of the back of SCID/beige mice. Plugs harvested at 3 weeks demonstrated that MU cells had generated myotubes, myofibers, and a unique spheroidal syncytia expressing a differentiated muscle phenotype (Figure 5E, desmin, MyHC, CD56). The myosac phenotype may result from spontaneous contraction of the myotube poorly adhering to the substrate and obviously not constrained by tendons. BM cells, while remaining viable, did not generate bone, cartilage, or muscle. CB cells, which formed bone and cartilage upon transplantation with an osteoconductive carrier, generated hyaline cartilage but not bone in Matrigel (Figure 5F).

MU CD146$^+$ Cells Are Perivascular, Committed Myogenic Progenitors

These data revealed a strong, spontaneous myogenic capacity specific for MU CD146$^+$ cells, fully consistent with the



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high expression of myogenic regulatory factors and genes reflecting the differentiated function of muscle (Figure S1B). Cell sorting, immunolocalization, and CFE assays identified MU CD146$^+$ cells as distinct from CD56$^+$/CD146$^-$ satellite cells and CD34$^+$/CD146$^+$ endothelial cells (Figure S4 and Tables S6A–S6C). In cultures established from highly purified CD56$^-$/CD146$^+$ cells, CD146 expression was turned off over time while expression of CD56, PAX7, ALP (previously reported to be a pericyte marker in muscle; Dellavalle et al., 2007], MyoD, Myogenin were turned on (Figures 6A and S1C). Only a small subset of clonogenic, myogenic CD146$^+$ cells co-expressed ALP (Figure 6B and Table S6D), and myogenic cells were highly enriched in the CD146$^+$/ALP$^-$ fraction (Figure 6C). In situ, ALP and CD146 were expressed in a mutually exclusive fashion in precapillary arterioles and postcapillary venules, respectively, suggesting that the venous compartment represents the largest repository of perivascular CD146$^+$ cells that can be assayed as myogenic progenitors (Figure 6D). Immunolocalization studies further revealed the presence of a relatively rare population of adventitial cells expressing muscle regulatory factor proteins (PAX3, PAX7) in postcapillary venules (Figure 6E). Freshly isolated MU CD146$^+$ cells also co-expressed PAX7 by FACS (Figure 6F) and by RT-PCR (Figure S1B).

Generation of Perivascular Cells from Different Types of Stromal Cells In Vivo

In heterotopic transplants made with osteoconductive carriers, BM-derived skeletal stem cells can guide and organize the formation of BVs, with which a subset of them ultimately associates (Sacchetti et al., 2007). To test the ability of BM- and non-BM-derived progenitors to guide and organize nascent BVs under simplified conditions that bar the formation of bone, we co-transplanted BM- and non-BM-derived progenitors in Matrigel, along with human umbilical vein endothelial cells (HUVECs). With cell-surface GFP-labeled BM-derived progenitors, this resulted in the formation of an extensive network of capillary-like BVs at 3 weeks. Their wall was composed of two cell layers: an inner hCD34$^+$ endothelial layer (red), and an outer, discontinuous layer of mural cells (green) made by transplanted BM progenitors, expressing CD146 and α-smooth muscle actin (α-SMA) (Figure 7A). In transplants harvested at 8 weeks, extensive maturation of the BV wall was observed, as indicated by the formation of thick-walled artery- and vein-like BVs, with a multilayered GFP$^+$, α-SMA$^+$ smooth muscle coat. The BVs were functional and perfused by host blood (Figure 7A). Experiments performed with cell-surface GFP-labeled MU CD146$^+$ cells also resulted in the formation of a capillary lattice, which was less extensive than with BM CD146$^+$ cells, but similarly well organized (Figure 7B, quantitated in Figure S5). No obvious differentiation of muscle structures were observed in these transplants, at variance with transplants of MU CD146$^+$ cells without HUVECs, suggesting that the contribution to BV formation and myogenesis could be alternative fates dictated by endothelial cells. With CB CD146$^+$ cells, again the formation of functional BVs was observed. Strikingly,

Figure 5. Intramuscular and Ectopic In Vivo Transplantation of CD146$^+$ "MSCs" Derived from Different Tissues

(A) Human BM (bone marrow), PE (periosteum), and CB (cord blood) derived cells in orthotopic transplantation into cardiotoxin (CTX)-injured muscle in SCID/beige mice after 4 weeks failed to contribute to generation of muscle cells or regenerating myofibers as would be detected by anti-human Lamin A/C (red) (laminin, green). Scale bar represents 75 μm.

(B) Human CD34$^-$/CD56$^-$/CD146$^+$ muscle-derived cells were injected intramuscularly into the left tibialis anterior of 2-month-old female SCID/beige mice, injured 1 day earlier by an intramuscular injection of CTX. After 4 weeks transplanted human MU cells, identified by expression of human Lamin A/C (red), were distributed to the interstitium (arrows), but also below the basement membrane (green) on the surface of myofibers (MF) in a satellite cell-like position (arrowheads). Scale bars represent 10 μm, 15 μm, or 20 μm as indicated.

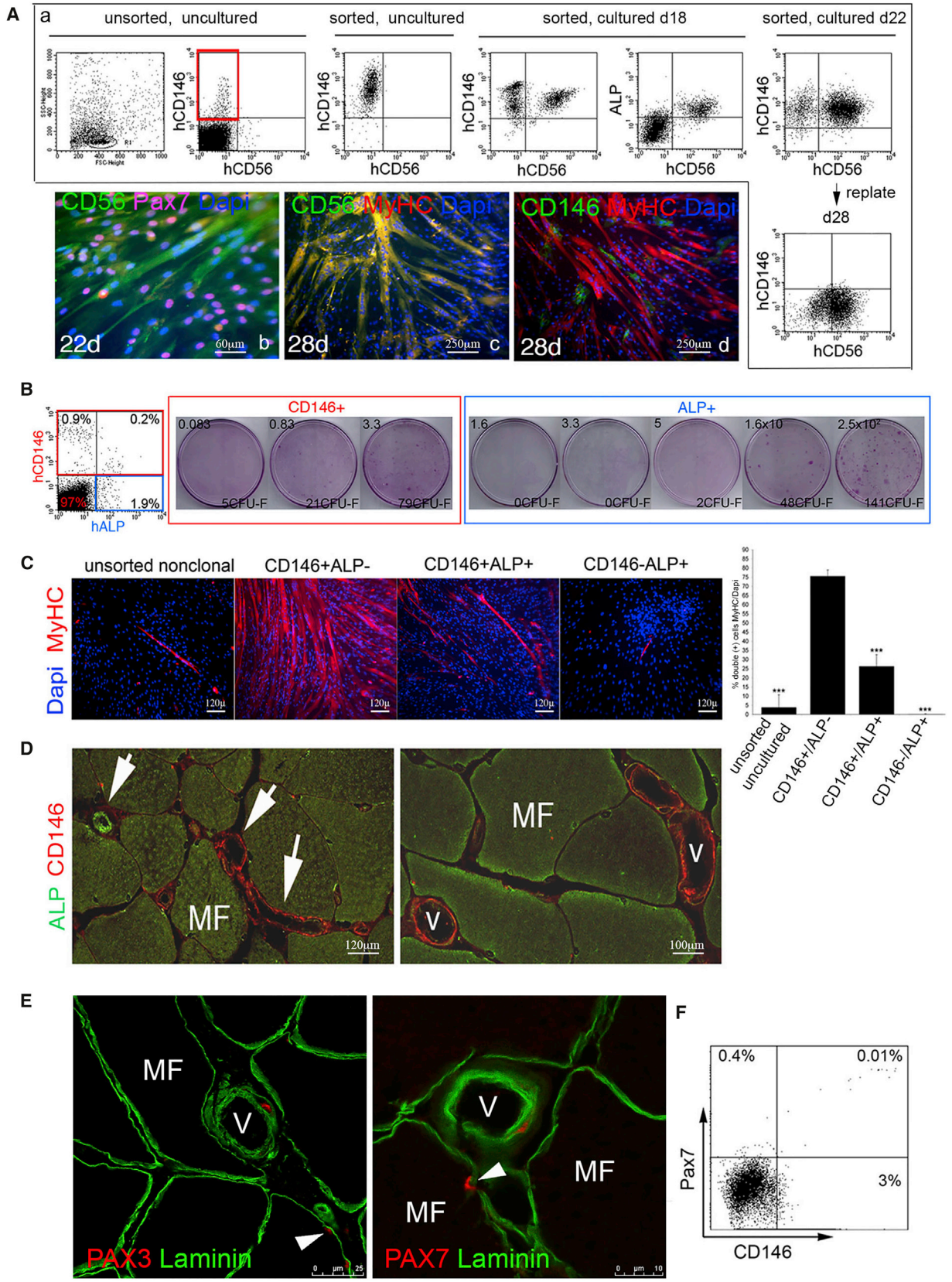
(C) Cells in a characteristic satellite cell-like position (arrowheads and arrow) express human CD56 (red). Scale bars represent 15 μm or 20 μm as indicated.

(D) Clusters of myofibers expressing human Dystrophin 2 and Spectrin were generated by MU cells by in vivo transplantation into SCID/beige/CTX-treated and SCID/mdx mice (DAPI: nuclear stain). Scale bar represents 350 μm.

(E) In a heterotopic transplantation assay, human CD34$^-$/CD56$^-$/CD146$^+$ MU cells, transduced to express a cell-surface GFP label, were suspended in growth factor-reduced Matrigel (labeled matrigel in panels) without HUVECs and injected into the epifascial space of the back of SCID/beige mice, and harvested 3 weeks later. Extensive formation not only of myotubes (a, b, and h) expressing human-specific myogenic markers, Desmin (g and k), myosin heavy chain (MyHC, h and i), and CD56 (j), but even of striated myofibers (c–e, black arrows) and non-conventional muscle structures (f, red arrows, i–k) were observed (H&E, a–f). Scale bars represent 5 μm, 10 μm, 15 μm, 25 μm, or 50 μm as indicated.

(F) No myogenic differentiation was observed with BM (stained blue with H&E in a, b, arrows) or any non-MU cell strain (lack of myotubes and myofibers). In contrast, CB CD146$^+$ "MSCs" spontaneously generated hyaline cartilage (stained light blue with H&E in c, d [arrows] and Alcian blue in e, f [arrows]) when transplanted in Matrigel as a carrier. No ingrowth of BVs into the Matrigel plug was associated with the extensive cartilage differentiation observed.

All data shown are representative results from one of at least three independent experiments. Scale bars represent 15 μm, 20 μm, 30 μm, 150 μm, or 200 μm as indicated.



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these BVs, lined by a continuous hCD34$^+$ endothelial layer, were coated by a thin layer of subendothelial CD146$^+$ mural cells, and an outer coat of differentiated chondrocytes (Figure 7C), making these BVs a unique kind of cartilage-armored BV, not known to occur in natural tissues. Knockdown of CD146 in cell-surface GFP-labeled BM progenitors (as previously described by Sacchetti et al., 2007) (Supplemental Experimental Procedures) partially interfered with BV organization, resulting in BVs of irregular size and shape, often devoid of a lumen, and devoid of a mural cell coat (Figure S6). Due to the ability of BM, MU, and CB cells to form perivascular cells, we examined the transcriptome data (including PE cells, although not used for this assay) for additional markers that are associated with pericytes (e.g., Armulik et al., 2011). A variety of pericyte markers were expressed by all four cell populations, but the pattern of expression varied from one cell type to another, consistent with their diverse developmental origins (Figure S7).

DISCUSSION

A widely accepted view in the literature holds that "MSCs" can be defined by rather loose and non-specific in vitro properties, and exhibit identical functional and differentiation properties regardless of their tissue source. This view, complemented by the notion that "MSCs" would coincide with ubiquitous pericytes, was recently reinforced by the claim that MU-derived pericytes would be both myogenic and skeletogenic, and exhibit an in situ surface phenotype similar to the one characteristic of BMSCs (i.e., of a skeletal stem cell origin) (Bianco, 2014). We have shown here that CD34$^-$/CD45$^-$/CD146$^+$ "MSCs" isolated from different tissues have inherently distinct transcriptomic signatures and differentiation capacities. By gauging their native differentiation potential with a variety of stringent differentiation assays, we demonstrated that human BM CD146$^+$ cells are inherently geared to generate bone and BM stroma that support hematopoiesis and include adipocytes, but are not myogenic, and are not spontaneously chondrogenic in vivo; MU "MSCs" with an identical cell-surface phenotype are not inherently skeletogenic, and are inherently myogenic; and CB "MSCs" are not myogenic, but are chondro-osteoprogenitors most likely due to their fetal origin (Bianco and Robey, 2015) and are the only kind of human "MSCs" ever shown to actually form cartilage consistently in open heterotopic transplants in vivo, independent of any ex vivo induction to cartilage differentiation as applied in the widely used pellet culture assay. CB cells are unlike other cells that are found in the umbilical cord, based on their cell-surface markers, their ability to differentiate into adipocytes and cartilage, and expression of associated markers, and they have a distinct expression pattern of

Figure 6. Induction of CD56 Expression, Colony-Forming Efficiency and Myogenic Differentiation of FACS Sorted Muscle-Derived "MSCs," and In Vivo Localization of ALP, CD146, and PAX 7

(A) Induction of the satellite cell marker, CD56, in cultures of CD34$^-$/CD56$^-$/CD146$^+$ cells by FACS and fluorescent immunocytochemistry. Red box indicates the population of CD146$^+$/CD56$^-$ cells used to establish the cultures. By FACS, co-expression of CD56 in a subset of CD146- and ALP-expressing cells is detectable after 18 days in culture, and the percentage of CD146$^+$/CD56$^+$ cells increases over time. Upon replating cultured CD146$^+$/CD56$^+$ cells at high density with 20% FBS, CD146 expression is turned off, whereas CD56$^+$ expressing cells remain detectable. By fluorescent immunocytochemistry, subconfluent cultures express CD56 (green) and PAX7 (purple when colocalized with the blue nuclear stain, DAPI) (22 days in culture), and when replated at high density with 20% FBS, myotubes with numerous nuclei (DAPI staining) express myosin heavy chain (MyHC, red), co-localizing with CD56 (green) resulting in yellow. At a terminal stage of differentiation (26–28 days in culture), CD146 expression is barely detectable. Scale bars represent 60 μm or 250 μm as indicated.

(B) FACS of collagenase-released cell suspensions of muscle. Co-expression of CD146 and ALP, in human MU cell suspensions before culture. CFE assay of FACS-sorted CD146$^-$/ALP$^+$ cells and CD146$^+$/ALP$^+$ cell subsets. CD146$^+$/ALP$^+$ and CD146$^-$/ALP$^+$ cells were plated at different cell densities. Numbers (upper left of each panel) indicate the number of cells plated/cm^2.

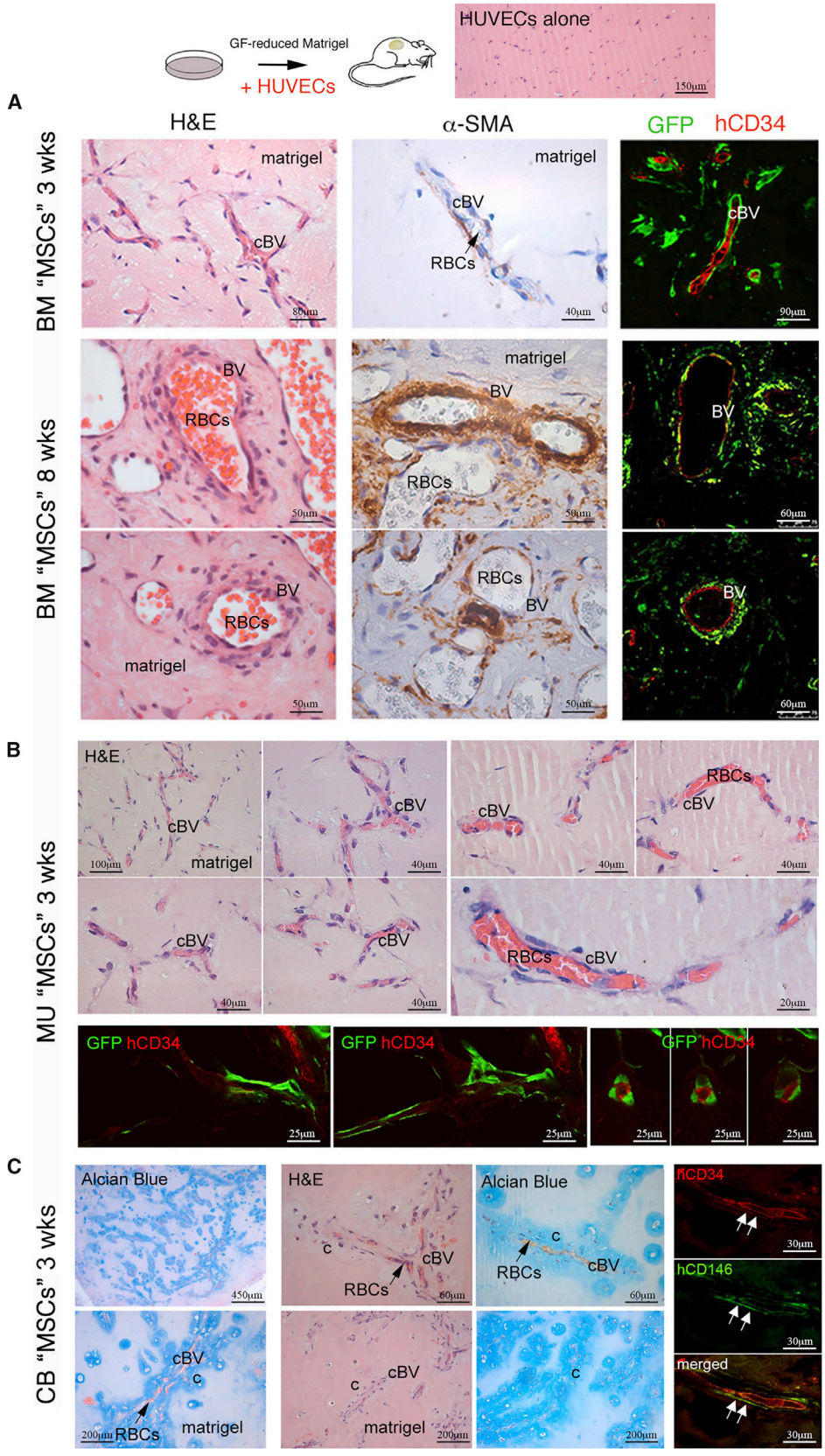
(C) When unsorted cell strains, sorted CD146$^+$/ALP$^-$, CD146$^+$/ALP$^+$, and CD146$^-$/ALP$^+$ total collagenase-released cells (bar graph is representative of one of at least three independent experiments, ***$p < 0.001$ compared with CD146$^+$/ALP$^-$ cells) were plated at clonal density and induced to myogenic differentiation (2% horse serum on Matrigel), high numbers of myotubes expressing skeletal muscle-specific myosin heavy chain (MyHC, red) are found in freshly sorted cultures enriched in MU CD146$^+$/ALP$^-$ cells. Only rare myotubes are observed in cultures of CD146$^+$/ALP$^+$ cells. Scale bar represents 120 μm.

(D) Immunolocalization analysis of the distribution of CD146 and ALP expression in intact muscle revealed that while ALP cells (green, arrow, upper left) were predominantly expressed in precapillary arterioles, CD146 cells (red, arrows, center and lower right) were predominantly expressed in large venules (v). Scale bars represent 100 μm or 120 μm as indicated.

(E) A subset of vascular-wall-associated cells was found to express PAX3 (red, left panel) and PAX7 (red, right panel) in intact adult skeletal MU. Immunoreactivity was distributed to the surface of myofibers (MF) in a satellite cell-like position (arrowhead) underneath the basement member (laminin, green), and to BV walls (V). Scale bars represent 10 μm or 25 μm as indicated.

(F) Freshly isolated, uncultured CD146$^+$ cells were found to co-express the satellite cell marker, PAX7.

All data shown are representative results from one of at least three independent experiments.



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HOX genes compared with other umbilical cord derivatives (Bosch et al., 2012; Liedtke et al., 2010, 2016). BM- and MU-derived clonogenic progenitors of mesoderm derivatives are associated with BV walls in situ and include cells in a position characteristic of mural cells/pericytes, but also include vascular-wall cells that do not necessarily exhibit anatomical features of pericytes proper, for example, adventitial cells in muscular veins. More strikingly, fetal chondro-osteoprogenitors that circulate in CB, by definition, are neither associated with BV walls nor represent pericytes. The origin of these cells remains to be elucidated. It is conceivable, however, that skeletal progenitors located at sites of active skeletal growth can accidentally spill over into the bloodstream. Nonetheless, independent of their origin, native differentiation potencies, gene-expression profiles, and in situ anatomical positioning, perinatal and postnatal mesoderm progenitors of different origins can dynamically associate with, and organize, nascent BVs as shown here using a simple in vivo assay.

Our data reveal that in lieu of a uniform, broadly multipotent, and equipotent class of ubiquitous "MSCs," perinatal and postnatal assayable progenitors found in CB, skeletal tissues, and skeletal muscle comprise a varied system of tissue-specific progenitors. Each member of the system is committed to a specific differentiation range. CB mesenchymal progenitors are committed to cartilage and bone formation, reminiscent of the potential of prenatal skeletal progenitors to generate skeletal rudiments made of cartilage and bone. Postnatal BM skeletal progenitors are instead committed to bone and BM stroma formation, but not to cartilage, mirroring an adult structure of skeletal segments. Myogenic and skeletogenic potential are mutually exclusive, as dictated by a specific set of master transcriptional regulators.

Our data are consistent with recent studies showing that in vivo, transplants of BM cells were able to form bone and support marrow formation, while white adipose-derived stromal cells (WAT), cells from umbilical cord (UC), and skin fibroblasts (SF) were not (Kaltz et al., 2008; Reinisch et al., 2015). Importantly, the "MSCs" derived from cord blood (CB) used in our study must be clearly distinguished from fibroblastic cells derived from UC "MSCs" in the Reinisch study; UC cells fail to differentiate in vitro and in vivo (Kaltz et al., 2008; Liedtke et al., 2016; Reinisch et al., 2015), differ in their typical *HOX* expression pattern (Liedtke et al., 2010, 2016), and have a different molecular transcriptomic signature lacking relevant integrin-binding sialoprotein (*IBSP*) expression (Bosch et al., 2012). Differences in our study include culture in 20% fetal bovine serum (FBS) and use of a ceramic carrier that favors direct osteogenesis (no evidence of endochondral bone formation). In Reinisch et al. (2015), BM cells were grown in 10% human platelet lysate and transplanted with a Matrigel equivalent (In Vitro Vasculogenesis Assay Kit; Millipore), and bone formed through an endochondral process. Another study investigated expression of μRNAs by BM cells, WAT cells, and CB cells at a multiclonal level (Ragni et al., 2013). While the μRNA patterns between the cells types were similar, a small number of highly differentially expressed μRNAs were identified, which could affect the expression of many genes. Of note, in vitro osteogenic, adipogenic, and chondrogenic differentiation assays were performed in both of these studies, and significant differences

Figure 7. In Vivo Transplantation of CD146$^+$ "MSCs" Derived from Different Tissues in Conjunction with HUVECs

(A) Co-transplant of cell-surface GFP-labeled BM-derived progenitors in Matrigel (labeled as matrigel) along with HUVECs resulted in the formation of an extensive network of capillary-like blood vessels (cBV) at 3 weeks as demonstrated by H&E staining. By fluorescence microscopy and immunostaining, their wall was made by two cell layers: an inner endothelial layer as marked by the endothelial marker, hCD34$^+$ (red), and an outer, discontinuous layer of mural cells made by transplanted, GFP-tagged BM progenitors. Transplants harvested at 8 weeks exhibited extensive maturation of the blood vessel (BV) wall as indicated by the formation of thick-walled artery- and vein-like BVs (H&E staining). These vessels were functional as indicated by perfusion with host blood, as shown by the presence of red blood cells (RBCs, black arrow). The vessels were coated with a multilayer of α-SMA$^+$ cells demonstrated by immunohistochemistry (middle column illuminated by differential interference contrast microscopy to see the disc-shaped host RBCs in the vessels). By fluorescence microscopy and immunostaining, a multilayer of GFP$^+$ cells surrounding hCD34$^+$ endothelial cells (red) were observed. Scale bars represent 40 μm, 50 μm, 60 μm, 80 μm, or 90 μm as indicated.

(B) Similar experiments with cell-surface GFP-labeled MU CD146$^+$ progenitors transplanted with HUVECs also resulted in the formation of a capillary lattice (cBV) at 3 weeks, which was less extensive than with BM cells, but similarly well organized and functional, as demonstrated by perfusion with host RBCs. As with BM cells, GFP-labeled MU cells were seen surrounding hCD34$^+$ endothelial cells (red). Scale bars represent 20 μm, 25 μm, 40 μm, or 100 μm as indicated.

(C) Heterotopic co-transplantation of CB cells in Matrigel along with HUVECs resulted in the formation of an extensive network of capillary-like BVs (cBV) at 3 weeks, which were functional and perfused by host RBCs (black arrows). By fluorescent immunohistochemistry, their wall was made by an inner continuous hCD34$^+$ endothelial layer (red, white arrows), coated by a subluminal layer of thin, hCD146$^+$ mural cells (green, white arrows) and an outer coat of chondrocytes and cartilage matrix (labeled c as identified by H&E [light blue staining] and the cartilage stain, Alcian blue), making these BVs a unique kind of cartilage-armored BV. Scale bars represent 30 μm, 60 μm, 200 μm, or 450 μm as indicated.

were noted between the cell types. Nonetheless, the authors of both papers concluded that an in vitro mesengenic process was at play in all cell types. However, in vitro analyses of osteogenic differentiation are not predictive of in vivo transplantation (e.g., Phillips et al., 2014). For chondrogenesis, 3D cultures are needed to clearly see bona fide chondrocytes lying in lacuna, surrounded by a matrix that stains purple with toluidine blue (metachromasia) as shown by Reinisch et al. (2015). Close examination of this study suggests that there was little or no chondrogenic differentiation of WAT, UC, or SFs compared with BM cells. In Ragni et al. (2013), chondrogenesis was difficult to assess based on Alcian blue-stained 2D cultures and expression of a limited number of markers, which is insufficient to confirm chondrogenic differentiation.

Even if updated to equate "MSCs" with pericytes (Caplan, 2008; Crisan et al., 2008), the widespread concept of broadly and uniformly multipotent "MSCs" that are invariant in anatomical space and developmental time leaves the developmental origin of such cells unaddressed, and in fact collides with certain fundamental tenets of developmental biology, such as the early segregation of inherent osteogenic and myogenic potential into different lineages (Bianco and Robey, 2015). Our data now reveal a simple mechanism whereby assayable skeletogenic or myogenic progenitors can be established in different tissues. As previously postulated (Bianco et al., 2008, 2013), we have now directly shown that committed progenitors of different origin and differentiation potential can physically associate with nascent BV walls in an experimental assay, and be recruited to a mural cell fate. In an earlier study, the most robust contribution of grafted mesoderm progenitor cells in quail chick chimeras was, in fact, to the adventitia of BVs of any caliber (Minasi et al., 2002). In development, somite-derived cells associate with, and are incorporated into, the wall of the dorsal aorta (Esner et al., 2006). Pericytes originate from the recruitment of non-endothelial, mesenchymal cells to vascular walls (Armulik et al., 2011; Hellstrom et al., 1999; Hirschi and D'Amore, 1996; Jain, 2003; Lindahl et al., 1997). Obviously, the same mechanism may operate in different tissues and at different developmental times. General mechanisms regulating mural cell recruitment have largely been elucidated, and provide the background against which to seek additional determinants operating in the retention of quiescent mesoderm progenitors in a vascular niche. CD146 itself may represent a player in this scenario, consistent with its nature as a cell-adhesion molecule (Shih, 1999), and with evidence provided here that its knockdown perturbs the establishment of properly structured BVs in vivo.

The direct interaction of committed progenitors with endothelial walls of nascent BV can be related to their arrested proliferation and differentiation, leading to retention, within specific tissues, of a compartment of progenitors with predefined commitment, and a potential for further growth defined by their replicative history prior to incorporation into BV walls. The long-known heterogeneity of clonogenic mesodermal progenitors (CFU-Fs) in proliferative potential ex vivo might find in this view a simple explanation.

Our data have additional implications of applied nature. Prospective isolation of putative "MSCs" from different tissues, based on the use of the minimal effective surface phenotype $CD34^-/CD45^-/CD146^+$ (Sacchetti et al., 2007), leads to isolation of committed tissue-specific progenitors, not of broadly multipotent, equivalent cells. Hence, design of cell-therapy strategies for specific tissues must remain cognizant of the specificity of the isolatable progenitors. In fact, reliance on this specificity leads to highly effective isolation of tissue-specific progenitors. This is best illustrated by the case of skeletal muscle, where we have shown that $CD146^+$ cells are far more enriched in spontaneously myogenic cells than previously identified populations of putative non-satellite cell classes (Dellavalle et al., 2007). Importantly, we have shown that these cells, which do express the satellite lineage marker, PAX7, and behave like satellite cells in in vitro and in vivo assays (Montarras et al., 2005), may in fact represent subsets of the same original population, recruited to distinct anatomical niches based on distinct, local cell-cell interactions, randomly directing individual progenitors to either the surface of developing fibers (satellite cells) or the surface of nascent BVs (myogenic pericytes, or else ectopic satellite cells). Consistent with this hypothesis, proximity of one anatomical locale to the other can amount, in fact, to less than one cell diameter. However, it is also possible that the pericytic MU $CD146^+$ cells are more primitive than satellite cells based on the fact that they give rise to $CD56^+$ satellite cells (Figure 5C). Also of note, MU $CD146^+$ cells do not coincide with PICs (interstitial populations of $PW1^+/PAX7^-$ cells; Mitchell et al., 2010), based on the fact that MU $CD146^+$ cells are $PAX7^+$, or FAPs (fibro/adipogenic progenitors) that cannot generate myofibers (Joe et al., 2010), while MU $CD146^+$ clearly do.

In summary, we have provided a rigorous analysis of mesodermal progenitors isolated from perinatal and postnatal tissues that reveals their inherent differentiation potential. These data underscore the fact that a ubiquitous "MSC" with identical differentiation capacities does not exist, and thus the "MSC" terminology should be abandoned for the sake of clarity. Furthermore, we have demonstrated that irrespective of their inherent differences, tissue-specific mesodermal progenitors are capable of being recruited to a mural cell fate, providing a plausible mechanism by which pericytes are formed, and how they serve as a source of local stem/progenitor cells.

EXPERIMENTAL PROCEDURES

Cell Isolation and Culture

Samples were obtained with informed consent according to institutionally approved protocols. BM-, PE-, and CB-derived cells, dermal fibroblasts, adipose-derived stromal cells, amniotic fluid cells, and HUVECs were grown as described in Supplemental Experimental Procedures.

For MU cells, normal muscle samples (1–30 × 10^2 mg) from 17 human adult patients (25–65 years old) were enzymatically dissociated as described in Supplemental Experimental Procedures. The resulting single-cell suspensions were used to obtain cultures of unfractionated cells, or for sorting of $CD146^+$ cells, $CD146^+/CD56^+$ cells, $CD146^+/CD34^+$ cells, or $CD146^+/ALP^+$ cells. Unsorted and sorted cells were plated in basal medium (α minimum essential medium [αMEM; Invitrogen] with 20% FBS [Invitrogen], 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin). For non-clonal cultures, cells were seeded at 1.6 × 10^3–1.6 × 10^5 cells/cm^2 in 75-cm^2 flasks or in 100-mm dishes (Becton Dickinson). For multiclonal cultures (combining multiple colonies), cells were seeded into 100-mm dishes at 1.6 cells/cm^2, and formation of discrete colonies was scored after 14 days. Non-clonal cultures were passaged when subconfluent; multiclonal cultures were passaged on day 14.

Cell Sorting and Flow Cytometry

Surface markers were assessed using a FACSCalibur flow cytometer with CellQuest software (BD Biosciences) as reported previously (Sacchetti et al., 2007) with mouse anti-human monoclonal antibodies (Table S7A) and isotype controls. $CD146^+$, $CD146^+/CD56^+$, $CD146^+/CD34^+$ cell subsets, and $CD146^+/ALP^+$ subsets were separated using a FACS DIVAntageSE flow cytometer (BD Labware). $CD146^+$, $CD146^+/CD56^+$, $CD146^+/CD34^+$, and $CD146^+/ALP^+$ fractions were separated using a MiniMACS magnetic column separation unit (Miltenyi Biotec) according to the manufacturer's instructions.

Gene-Expression Profiling and Data Analysis

Total RNA was isolated from multiclonal cultures of $CD146^+$ cell populations from three independent cultures after 2 weeks of culture in basal medium (see above), and processed as described in Supplemental Experimental Procedures. Raw data of gene-expression profiling were submitted to the GEO repository (GEO: GSE69991).

Gene Set Enrichment Analysis

GSEA was performed using GSEA software (http://www.broadinstitute.org/gsea/index.jsp) (Subramanian et al., 2005) as described in Supplemental Experimental Procedures.

Colony-Forming Efficiency Assays

CFE assays were performed with different cell fractions obtained by cell sorting. $CD146^+$ cells were seeded in basal medium (see above) at 1.6–3.3 cells/cm^2, $CD146^+/CD34^+$ and $CD146^+/CD56^-$ cells were seeded at 1.6–8.3 cells/cm^2, and $CD146^+/ALP^+$ were seeded at 0.083–3.3 cells/cm^2. $CD146^-$ ($CD56^+$ or $CD34^+$ or ALP^+) unsorted cells were seeded at an equivalent or higher density (1.6–1.6 × 10^5 cells/cm^2) and grown under identical conditions. After 14 days, CFE was determined as the mean number ± SD of colonies (>50 cells)/10^2–10^5 cells initially plated. ALP cytochemistry was done using naphthol-AS-phosphate as substrate and Fast Blue BB as coupler.

In Vitro Differentiation Assays

Spontaneous myogenic differentiation was assessed by plating cells onto Matrigel-coated dishes, with DMEM/2% horse serum, or on plastic with αMEM/20% FBS at clonal density. After 7 days, cultures were fixed and labeled for immunofluorescence with a monoclonal antibody against striated MyHC. Myogenic efficiency was estimated as the percentage of $DAPI^+$ nuclei found within myosin-positive myotubes. Fluorescence images were obtained using an Eclipse TE2000 Inverted Microscope (Nikon). Data were compared by ANOVA.

In Vivo Transplantation Assays

All animal procedures were approved by the relevant institutional committees.

Heterotopic Bone Formation

Constructs of test cells and osteoconductive material (hydroxyapatite/tricalcium phosphate [HA/TCP; Zimmer]) were transplanted into the subcutaneous tissue of SCID/beige mice (CB17.Cg-*Prkdc*^{scid}*Lyst*$^{bg\text{-}J}$/Crl; Charles River), using an established assay (Krebsbach et al., 1997; Sacchetti et al., 2007).

Orthotopic Myogenesis

CTX model: MU- BM-, and CB-derived cell populations (1 × 10^6) were injected intramuscularly into the left tibialis anterior of 2-month-old female SCID/beige mice injured 1 day earlier by an intramuscular injection of cardiotoxin (CTX; Latoxan) (Dellavalle et al., 2007). Muscles were examined 4 weeks following transplantation. SCID/mdx model: MU- and BM-derived cell populations were injected via the femoral artery of 2-month-old female SCID/*mdx* dystrophic mice (C57BL/10ScSn-*mdx*/J; Jackson Laboratory) as described by Dellavalle et al. (2007). Two injections of 5 × 10^5 cells at a 15-day interval were performed, and animals were euthanized 15 days after the last injection (30 days in total). The injected tibialis anterior muscles were analyzed for myogenic markers by immunofluorescence, with uninjected contralateral muscles serving as controls.

Heterotopic Differentiation, Matrigel

1 × 10^6 cells from multiclonal cultures of MU, BM, and CB cells were suspended in 1 ml of Matrigel Growth Factor-Reduced (BD Biosciences Labware), either alone or mixed with an equal number of HUVECs (Cambrex). Aliquots (~0.7 ml) were injected in the subcutaneous tissue of the back of SCID/beige mice, and transplants were harvested after 20 days. In some experiments, cells from multiclonal cultures of MU and BM cells were transduced with GFP-lentiviral vectors.

Lentiviral Vectors

Lentiviral vectors for GFP expression and CD146 silencing were produced and used as described previously (Piersanti et al., 2010; Sacchetti et al., 2007). Sorted $CD146^+$ cells from BM and MU were transduced with GFP-lentiviral vectors at an MOI of 5 and cultured

for 2 weeks in basal medium (see above) before use. CD146 silencing is described in Supplemental Experimental Procedures.

Immunohistochemistry Studies

Heterotopic and orthotopic transplants were processed as described in Supplemental Experimental Procedures. All experiments using mice were performed under institutionally approved protocols. All primary antibodies used for immunolocalization studies are listed in Table S7B.

RT-PCR

Conditions used in this study are described in Supplemental Experimental Procedures, and primers are listed in Table S7C.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and seven tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2016.05.011.

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Supplemental Information

No Identical ``Mesenchymal Stem Cells" at Different Times and Sites: Human Committed Progenitors of Distinct Origin and Differentiation Potential Are Incorporated as Adventitial Cells in Microvessels

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SUPPLEMENTAL INFORMATION

EXPERIMENTAL PROCEDURES, REFERENCES, FIGURE LEGENDS AND FIGURES

No identical "mesenchymal stem cells" at different times and sites: Human committed progenitors of distinct origin and differentiation potential are incorporated as adventitial cells in microvessels

Benedetto Sacchetti, Alessia Funari, Cristina Remoli, Giuseppe Giannicola, Gesine Kogler, Stefanie Liedtke, Giulio Cossu, Marta Serafini, Maurilio Sampaolesi, Enrico Tagliafico, Elena Tenedini, Isabella Saggio, Pamela G. Robey, Mara Riminucci, Paolo Bianco

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell isolation and culture

BMSCs were isolated and cultured as previously described (Sacchetti et al., 2007) from surgical waste from long bones, or iliac crest bone marrow aspirates. PE cells were generated as per established methods (Cicconetti et al., 2007). Human CB cells (≥36wks of gestation) were isolated and cultured as described previously (Kluth et al., 2010). Purchased human dermal fibroblasts (PromoCell GmbH, Heidelberg, Germany) were cultured in DMEM-high glucose (Invitrogen), supplemented with 2mM glutamine. Human amniotic cells were isolated as previsouly described (Pievani et al., 2014). HUVECs were grown in Clonetics EGM-2 BulletKit (Cambrex Corporation) following the manufacter's instructions.

Muscle-derived cells were isolated from normal skeletal muscle (1-30x10^2mg) from 17 human adult patients (aged 25-65 yrs) undergoing orthopedic surgery [*vastus lateralis* (1), *quadriceps femoris* (5), *triceps brachii* (2), *deltoides* (4), *gluteus maximus* (5)]. Samples were washed with Hank's balanced salt solution without Ca^{2+}/Mg^{2+} (HBSS, Invitrogen Life Technologies Corp) containing 30mM HEPES (Sigma), 100U/ml penicillin, 100μg/ml streptomycin (Invitrogen) for 10min at room temperature with gentle agitation. Tissue samples were used to obtain single cell suspensions by digesting twice with 100U/ml *Clostridium histolyticum* type II collagenase (Invitrogen) supplemented with 3mM $CaCl_2$ in

Ca^{2+}/Mg^{2+}-free PBS (Invitrogen) for 40 min at 37°C with gentle agitation. The samples were centrifuged at 1000 rpm for 5min at 4°C, washed with Ca^{2+}/Mg^{2+}-free PBS, resuspended in PBS, passed through 18 gauge needles to break up cell aggregates, and filtered through a 40μm pore size cell strainer (Becton Dickinson) to obtain a single cell suspension. Nucleated cells were counted using a haemocytometer.

Human adipose tissue-derived cells were obtained from human adult subcutaneous adipose tissue. Fat tissue was minced with scissors, washed with Ca^{2+}/Mg^{2+}-free PBS and the extracellular matrix was digested with collagenase type I (Invitrogen), at 37°C for 1h. The samples were centrifuged at 1000 rpm for 5min at 4°C, washed with Ca^{2+}/Mg^{2+}-free PBS, resuspended in PBS, passed through 18 gauge needles to break up cell aggregates, and filtered through a 40μm pore size cell strainer to obtain a single cell suspension.

RT-PCR Analysis

From CD146-sorted, uncultured cells, total RNA was extracted using a PicoPure™ RNA Isolation Kit (Arcturus Bioscience), per the manufacturer's instruction. cDNA was synthesized using 9μl of RNA, 100ng of random hexamers, and 50u of SuperScriptII Reverse Transcriptase (Invitrogen) in a total volume of 20μl. From cultured cells, total RNA was extracted using TRIZOL™ RNA isolation system (Invitrogen) per the manufacturer's instructions. cDNA was synthesized using 3μg of RNA, 150ng of random hexamers, and 50u of SuperScript II Reverse Transcriptase (Invitrogen) in a total volume of 20μl. Target cDNA sequences were amplified in standard PCR reactions using Platinum® PCR SuperMix according to the manufacturer's instructions. Primers used for RT-PCR are described in Supplemental Table 7C.

Gene expression profiling and data analysis.

Total RNA was isolated from multi-clonal cultures of $CD146^+$ cell populations after 2wks of culture in basal culture conditions (αMEM (Invitrogen) with 20% FBS (Invitrogen), 2mM L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin) using RNeasy RNA isolation kit (Qiagen) per the manufacturer's instructions. Disposable RNA chips (Agilent RNA 6000 Nano LabChip kit) were used to determine the concentration and purity/integrity of RNA samples using an Agilent 2100 bioanalyzer. cDNA synthesis, biotin-labeled target synthesis, HG-U133 plus 2.0 GeneChip (Affymetrix) array hybridization, staining and scanning were performed according to the standard protocol supplied by Affymetrix. Probe level data were normalized and converted to expression values using Partek Genomics Suite 6.2 (Partek Inc), following the RMA algorithm (Irizarry RA, et al. 2003) or DChip procedure (invariant set) (Li and Wong, 2001; Li and Wong, 2001). Quality control assessment was performed using different Bioconductor packages such as R-AffyQC Report, R-Affy-PLM, R-RNA Degradation Plot and Partek's QC. Low quality samples were removed from analysis. Before significance analysis, Partek's batch correction method, which reduces variation due to random factors, was used to enhance signal. Sample data were then filtered in order to remove probesets having a standard deviation/mean ratio greater the 0.8 and less that 1000. Principal Component Analysis (PCA) as well as the unsupervised hierarchical clustering were performed using Partek GS®. The agglomerative hierarchical clustering was performed using the Euclidean distance and the average linkage method. Differentially expressed genes were selected using a supervised approach using the ANOVA package included in Partek GS® Software. Formally, an unpaired t-test using a contrast fold change of at least 3 and an FDR (q-value) <0.05 was used in order to perform multiple pairwise comparisons between each class and the rest. Raw data of gene expression profiling were submitted to GEO repository (GSE69991).

Gene Set Enrichment Analysis

Gene Set Enrichment Analysis (GSEA) was performed using GSEA software [(http://www.broadinstitute.org/gsea/index.jsp; (Subramanian et al., 2005)] on log2 expression data of $CD146^+$ cell population purified from BM, CB, MU and PE and classified in the corresponding classes. Gene sets were taken from the Molecular Signatures Database (http://www.broadinstitute.org/gsea/msigdb/index.jsp). In particular, we investigated whether each class of $CD146^+$ cells was associated with over- or under-represented genes in pairwise comparisons between each class and the rest. Gene sets significantly over- or under-represented were returned by GSEA as showing an Enrichment Score ES<0 and an FDR<25% when using Signal2Noise as metric and 1,000 permutations of phenotype labels.

Secondary Passage of $CD146^+$ MU CFU-Fs (Self-renewal)

Muscle $CD146^+/CD34^-$ cells were injected i.m. into the left *tibialis anterior* as described in the text. At 6wks, animals were euthanized and the injected and contralateral (control) *tibialis anterior* were harvested, washed with Ca^{2+}/Mg^{2+}-free PBS and the tissue was digested with collagenase type II (Invitrogen) at 37°C for 1h. The samples were centrifuged at 1100rpm for 5min at 4°C, washed with Ca^{2+}/Mg^{2+}-free PBS, resuspended in PBS, passed through 18 gauge needles to break up cell aggregates, and filtered through a 40μm pore size cell strainer to obtain a single cell suspension. ~$5x10^5$ cells were obtained from the digestions. For flow cytometry, dissociated cells were incubated with mouse monoclonal antibodies specific for human CD146, human CD44 and human CD90. The isolated human cells were plated in culture and analyzed by FACS at 2wks for expression of hCD146, hCD90, and hCD44. Human cells were magnetically separated based on CD44 and CD90 expression using MiniMacs (Miltenyi). To assay for secondary MU-CFU-Fs, positive cells were recovered and resuspended in medium; cells were plated in culture at clonal density (1.6 cells/cm^2) and scored for colony formation at

2wks. The discrete colonies obtained were harvested and analyzed by FACS for expression of hCD56, hCD146 and hCD34.

Immunohistochemistry studies

Orthotopic and heterotopic transplants were snap-frozen in OCT embedding medium in liquid nitrogen and cryostat-sectioned serially, or alternatively fixed in 4% formaldehyde (and decalcified in the case of bone- or ceramic-containing transplants) and processed for paraffin embedding. Five-μm thick paraffin sections were stained with H&E, Safranin O and Light Green, Alcian blue or Sirius red for histology. All primary antibodies used for immunolocalization studies are listed in Supplemental Table 13 and were used as per standard immunoperoxidase (DAB reaction) or immunofluorescence protocols. Secondary antibodies labeled with Alexa Fluor 594 and 488, were from Molecular Probes (Invitrogen). Nuclei were stained by DAPI or Propidium Iodide (Sigma). Fluorescence images-stacks were obtained using confocal microscopy laser scanning (Leica TCS SP5, Leica Microsystems). Brightfield light and polarized light microscopy images were obtained using Zeiss Axiophot microscope (Carl Zeiss)**.**

Microvessel density analysis

Microvessel density analysis was performed as described (Melero-Martin J.M. et al., 2008). Microvessels were quantified by evaluation of 10 randomly selected fields of H&E stained sections taken from the transplants. Microvessels were identified as lumenal structures containing red blood cells and counted. Microvessels density was reported as the average number of red blood cell filled microvessels from the fields analyzed and expressed as vessels/mm^2. Values reported for each experimental condition correspond to the average values ± S.D. obtained from at least three individual mice.

Knockdown of CD146 in BM progenitors

Short hairpin (sh) sequences (19nt) targeted to human CD146 exon 6, 8 and 15 were designed using algorithms in the public domain (http://www.ambion.com/techlib/misc/siRNA_finder.html), submitted to BLAST analysis to exclude off-target annealing, and custom-synthesized (Operon Biotechnologies GmbH, Cologne, Germany). The control 19nt sequence was designed to not match any sequence in the human genome. The shRNA duplexes were cloned into ClaI/MluI sites of the pLVTHM-eGFP lentiviral transfer vector (from D. Trono, Ecole Politechnique, Genève Switzerland; maps at http://www.tronolab.com), downstream of the H1 promoter. Lenti-viral vectors were produced as described (Piersanti et al., 2006), by transfecting 293T cells with the transfer vector, the packaging vector pCMV-dR8.74 and the VSV-G envelope vector pMD2G (http://www.tronolab.com). BMSCs were infected with each lentivirus as described (Piersanti et al., 2006). Efficiency and efficacy were assessed by western blot analysis and FACS (CD146). The lenti-viral vectors encoding shRNA targeted to CD146 exon 15 (LV-shCD146) was chosen as the most effective and used or experiments at an MOI of 1.

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SUPPLEMENTAL FIGURES

Supplemental Figure 1. Transcriptome, RT-PCR and fluorescent immunocytochemistry analyses of myogenic markers in "MSCs." A) Analysis of the transcriptome of BM (bone marrow), CB (cord blood), MU (muscle) and PE (periosteum) cells shows signification expression of PAX7 only in MU cells (circles – replicates, boxes – average $\pm$ SD). Results are derived from 3 independent cultures of each cell type. B) RT-PCR analysis of myogenic regulators and markers in "MSCs," demonstrating restriction of their expression to $CD146^+$ muscle-derived cells only (sorted a and b – two independent populations of cells). C) Sorted and cultured cultures $CD146^+/CD56^-$ muscle-derived cells progressively turn on expression of human-specific myogenic markers PAX3, Myf5 and Desmin. Results for (B) and (C) are representative results from 1 of at least 3 independent experiments.

Supplemental Figure 2. Transcriptome analysis for hematopoietic cytokines, and in vivo transplantation of "MSCs." A) Expression of hematopoiesis-supportive factors by BM (bone marrow)-, CB (cord blood)-, MU (muscle)- and PE (periosteum)-derived cells. Results are derived from 3 independent cultures of each cell type. Hematopoietic factors are highly over expressed in BM cells compared with the others.

In vivo differentiation of bone marrow-(B), dermis-(C), adipose-(D) and amniotic fluid-derived cells (E). When cultured "MSC" cell strains were transplanted, using the same *in vivo* assays and HA/TCP as a carrier and stained with Sirius red, osteogenic potential was restricted to bone marrow-derived cells (BM). Cells derived in culture from dermis (D), adipose tissue (A) or amniotic fluid (AF), regularly failed to form any histological bone, whereas "MSCs" from BM did form bone and establish the hematopoietic microenvironment *in vivo* (* in panel B) (Bars=300μm).

Supplemental Figure 3. Self-renewal of MU $CD146^+$ cells. Muscle $CD146^+/CD34^-$ cells were injected i.m. into the left *tibialis anterior*. At 6 wks, animals were euthanized and the injected and contralateral (control) *tibialis anterior* were harvested. Subsequently, collagenase-released cells of the harvested injected TA were used to perform secondary MU colony forming efficiency assays. Human cells were isolated based on hCD44, hCD90 and hCD146 expression and after brief expansion (2 wks), reanalyzed by FACS for hCD44, hCD90 and hCD146 expression. The human cells were the isolated by MiniMacs (Miltenyi), and were replated in culture at clonal density. All colonies harvested at 2 wks were uniformly positive for hCD146 and negative for hCD56 (a mature myogenic marker) and hCD34 (a hematopoietic/endothelial marker), demonstrating that $hCD146^+$ pericytes isolated from the injected muscle were the source of the secondary MU-CFU-Fs, indicative of self-renewal. Results are representative of 1 out of 2 independent experiments.

Supplemental Figure 4. In MU cells, expression of CD146, CD56 and CD34 by FACS fluorescent, immunohistochemistry and immunocytochemistry, and colony forming efficiency. A) Isotype control and dual label FACS analysis of a collagenase-released muscle cell suspension. Expression of CD146 and CD56 is mutually exclusive in distinct cell subsets, with no co-expression. B) Localization of CD146 and CD56 in muscle sections. CD56 is restricted to the surface of myofibers where satellite cells reside

(*arrows* in b, *red arrows* in e). CD146 is restricted to vascular walls (*arrows* in d, *green arrows* in e). MF, myofiber. Bars=70μm in a-d, 20μm in e. C) CFE assay with $CD146^{\pm}/CD56^{\pm}$ subsets of collagenase-released cells. CFU-Fs capable of growth on plastic are found in the $CD146^{+}/CD56^{-}$ fraction. Numbers (upper left each panel) indicate the number of cells plated/cm^2. D) Isotype control and dual label FACS of CD146 and CD34 expression in collagenase-released muscle cells. ~25% of CD146+ cells co-express CD34 (ellipse), and ~12% of CD34+ cells express CD146. E) Localization of CD146 and CD34 in muscle sections. a) Both antigens are localized to cross-sections of pre-capillary arterioles and post-capillary venules (a, *large arrows*), whereas most capillaries (a,i,j, *small arrows*) among myofibers (MF) only label for CD34. b-d) Detail of a large pre-capillary arteriole. Endothelial cells [*e* in d] co-express CD34 and CD146; subendothelial mural cells [*peri* in d] only express CD146. e-g) Detail of a small pre-capillary arteriole. Endothelial cells express CD34 but not CD146. Subendothelial cells express CD146 but not CD34. h-j) Detail of a capillary adjacent to a myofiber (MF). Endothelial cells express CD34 but not CD146. No CD146 expression is detected. Bar=90μm in a; 50μm in b-g; 10μm h-j. F) When sorted $CD146^{+}/CD34^{-}$, $CD146^{+}/CD34^{+}$ and $CD146^{-}/CD34^{+}$ total collagenase-released cells plated at clonal density are induced to myogenic differentiation (2% HS on Matrigel™), high numbers of myotubes expressing skeletal muscle-specific myosin heavy chain (MyHC) are found in freshly sorted cultures enriched in muscle-derived $CD146^{+}/CD34^{-}$ cells. Only rare myotubes are observed in $CD146^{+}/CD34^{+}$ (Bars=100μm or 120μm).

Supplemental Figure 5. Formation of vascular networks by "MSCs" *in vivo*. MU (a,d), CB (b,e) and BM (c,f) cells with HUVECs (a-c) or alone (d-f) were resuspended in Matrigel and implanted on the backs of SCID/beige mice by subcutaneous injection. Implants were harvested after 21 days and stained with H&E. H&E staining revealed the presence of lumenal structures containing erythrocytes (black arrow heads) in implants where both cells types (HUVECs and MU, CB or BM cells) were used (a,b,c)

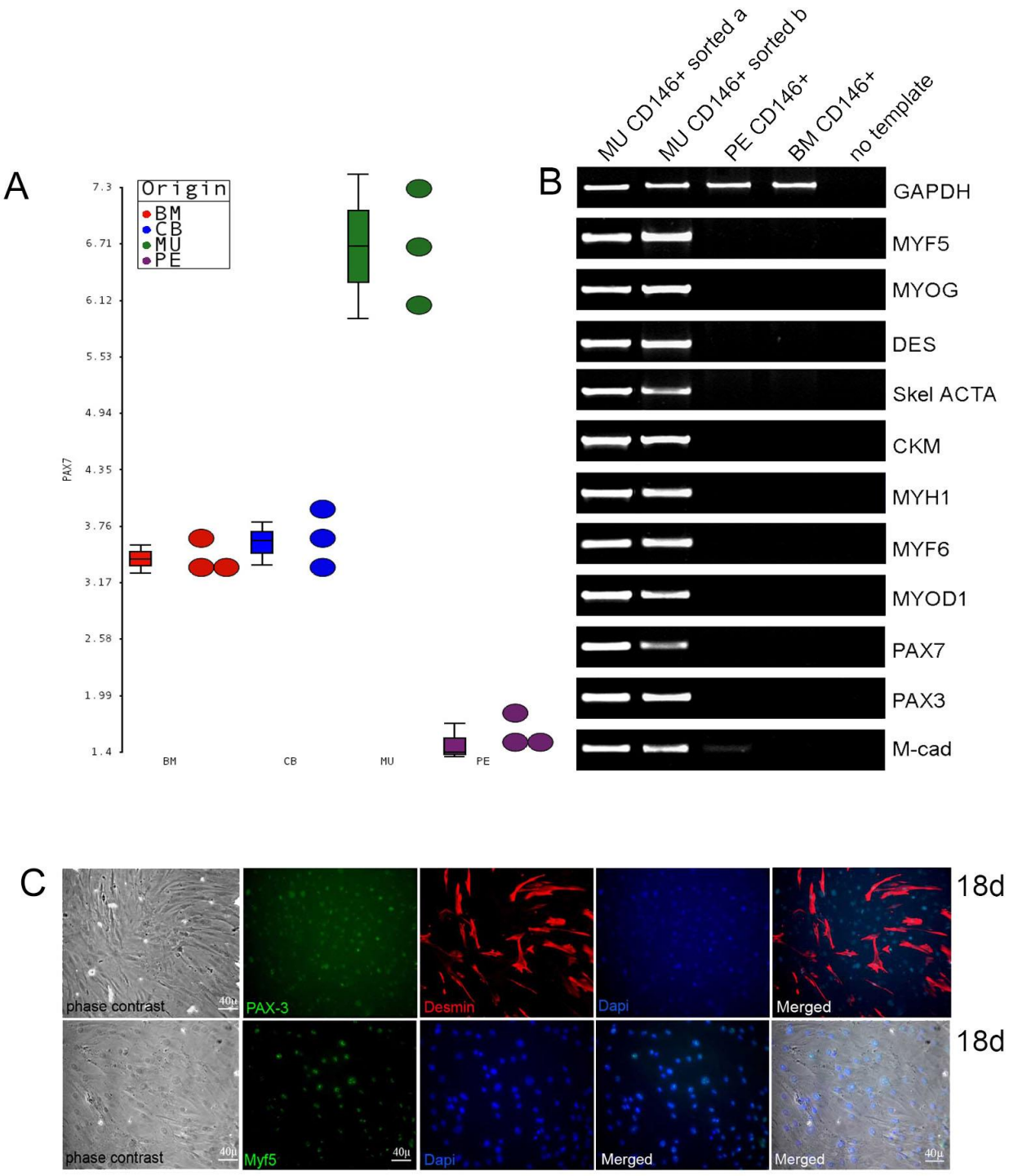
but not in implants where MU (d), CB (e), or BM (f) were used alone (Bars=100 μm). Images are representative of implants harvested from at least 3 different mice. Quantification of microvessel density was performed by counting erythrocyte-filled vessels in implants (g; n=3 each condition). Each bar represents the mean±SD (vessels/□m^2) obtained from the vascularized implants, *$p<0.01$. Results are from 1 experiment representative of at least 3 independent experiments.

Supplemental Figure 6. Effect of knockdown of CD146 in BMSCs in vessel formation with HUVECs in an *in vivo* transplantation assay. A) Co-transplant at 3 wks of BM-derived progenitors (green, cell-surface GFP) transduced with a control lentiviral shRNA (LVsh-Ctr) in Matrigel™, along with HUVECs (red, hCD34) resulting in the formation of a well organized capillary lattice. One can clearly see that the structures formed have a lumen (*) created by endothelial cells that are surrounded by BM cells (arrows). B) Co-transplant at 3 weeks of in BM-derived progenitors in which CD146 was knockdowned by shRNA (LVshCD146) in Matrigel™, along with HUVECs. In this case, structures that are formed are of irregular size and shape (arrows, compare with panel A). In many instances, they were devoid of a lumen (**), and devoid of a regular mural cell coat (arrows), or completely disorganized (arrow heads). In many instances, there is no association between the endothelials cells and the BM cells at all (Bars=20μm, 80μm, 90μm, 130μm or 150μm). Results are from 1 experiment representative of at least 3 independent experiments.

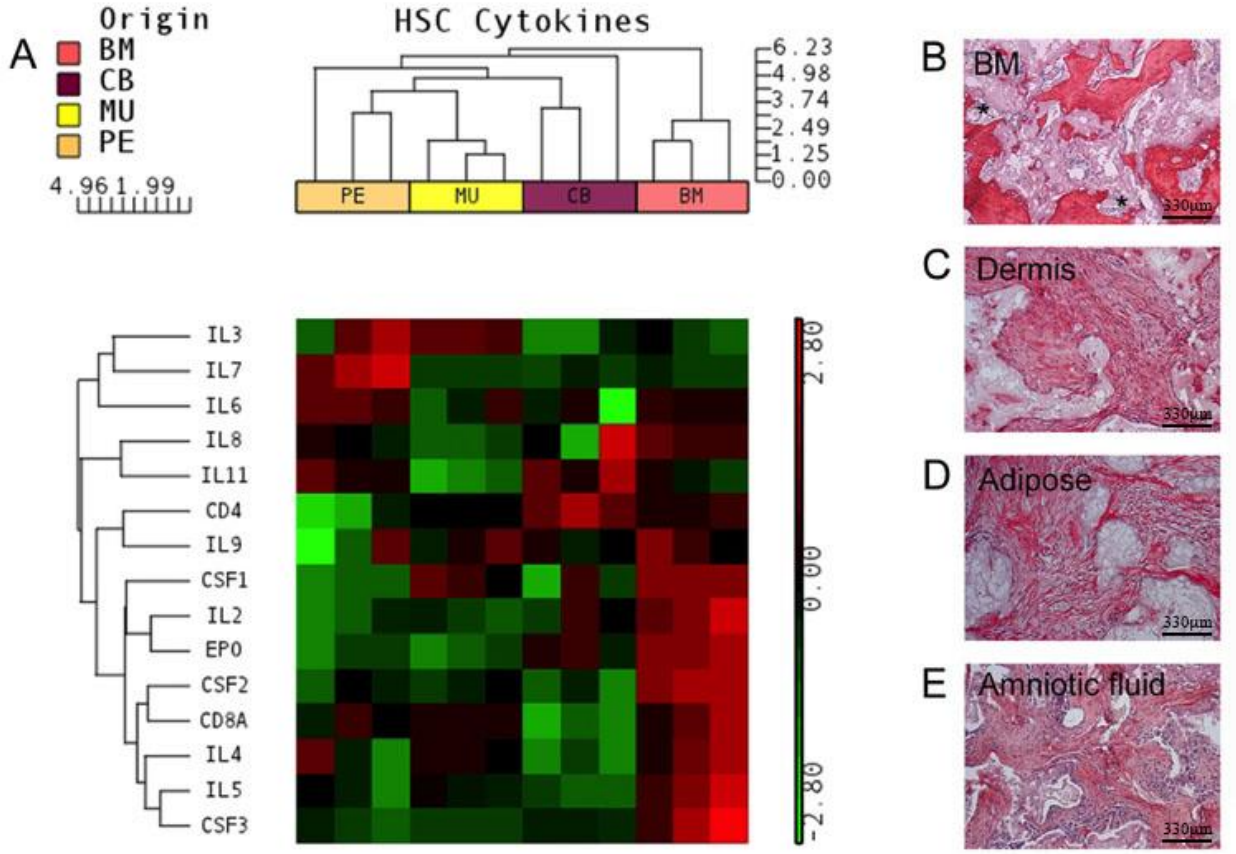
Supplemental Figure 7. Comparison of pericyte-related genes expressed by "MSCs." BM (bone marrow), MU (muscle), CB (cord blood) and PE (periosteum) cells expressed several pericyte-related genes: however, no cell type expressed all of them, and the pattern of expression varied by cell type,

consistent with their diverse developmental origins. Results were derived from the analysis of 3 indpendent cultures for each cell type.

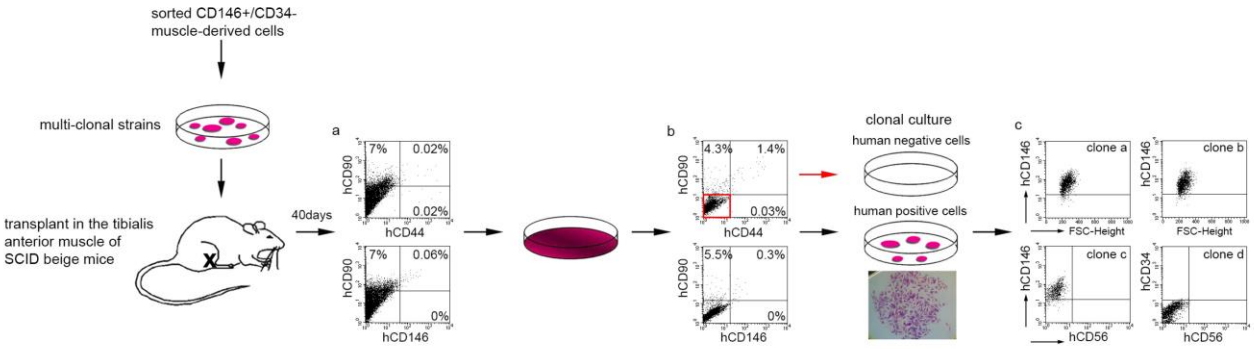
Supplemental Figure 1. Transcriptome, RT-PCR and fluorescent immunocytochemistry analyses of myogenic markers in "MSCs."



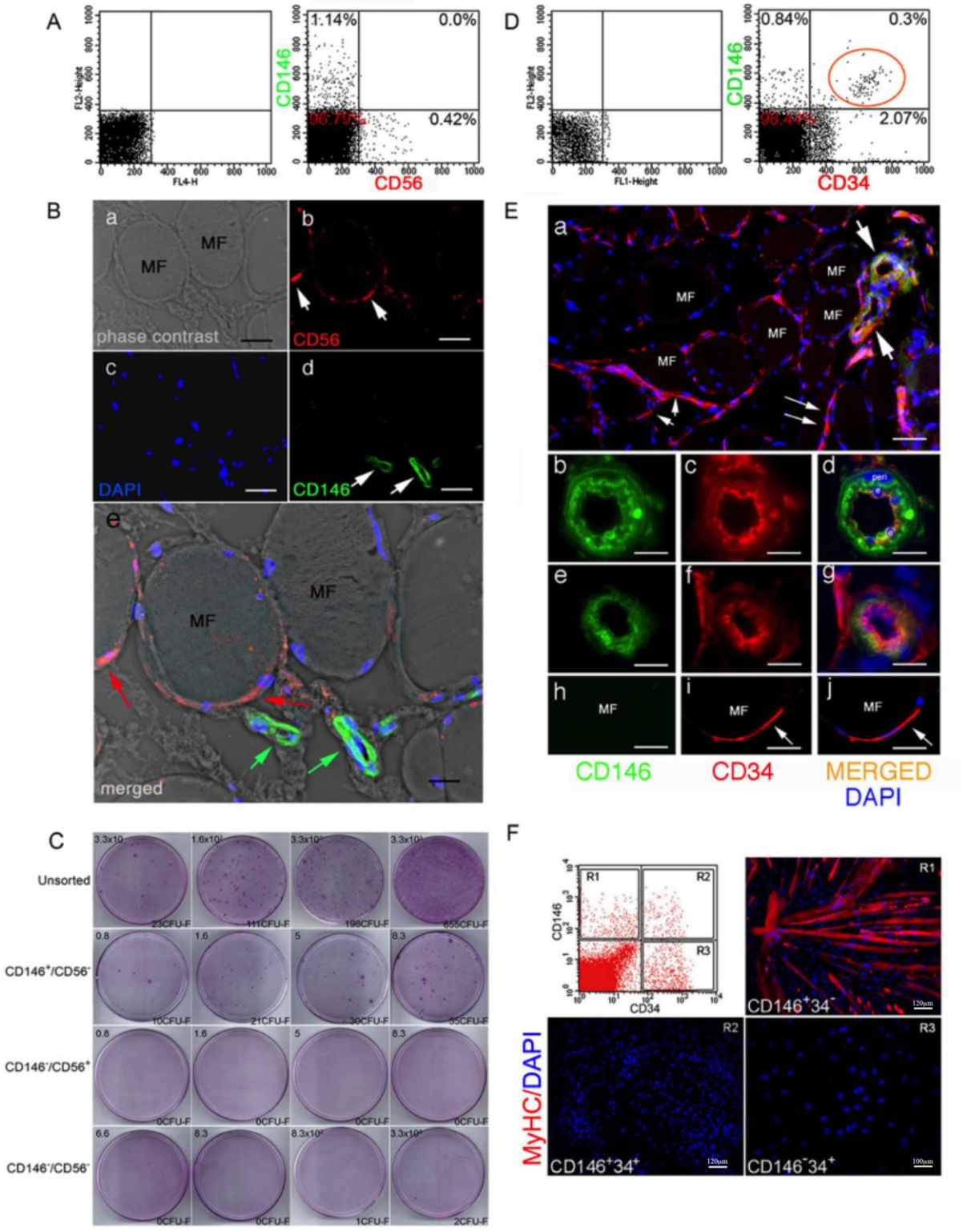
Supplemental Figure 2. Transcriptome analysis for hematopoietic cytokines and in vivo transplantation of "MSCs."



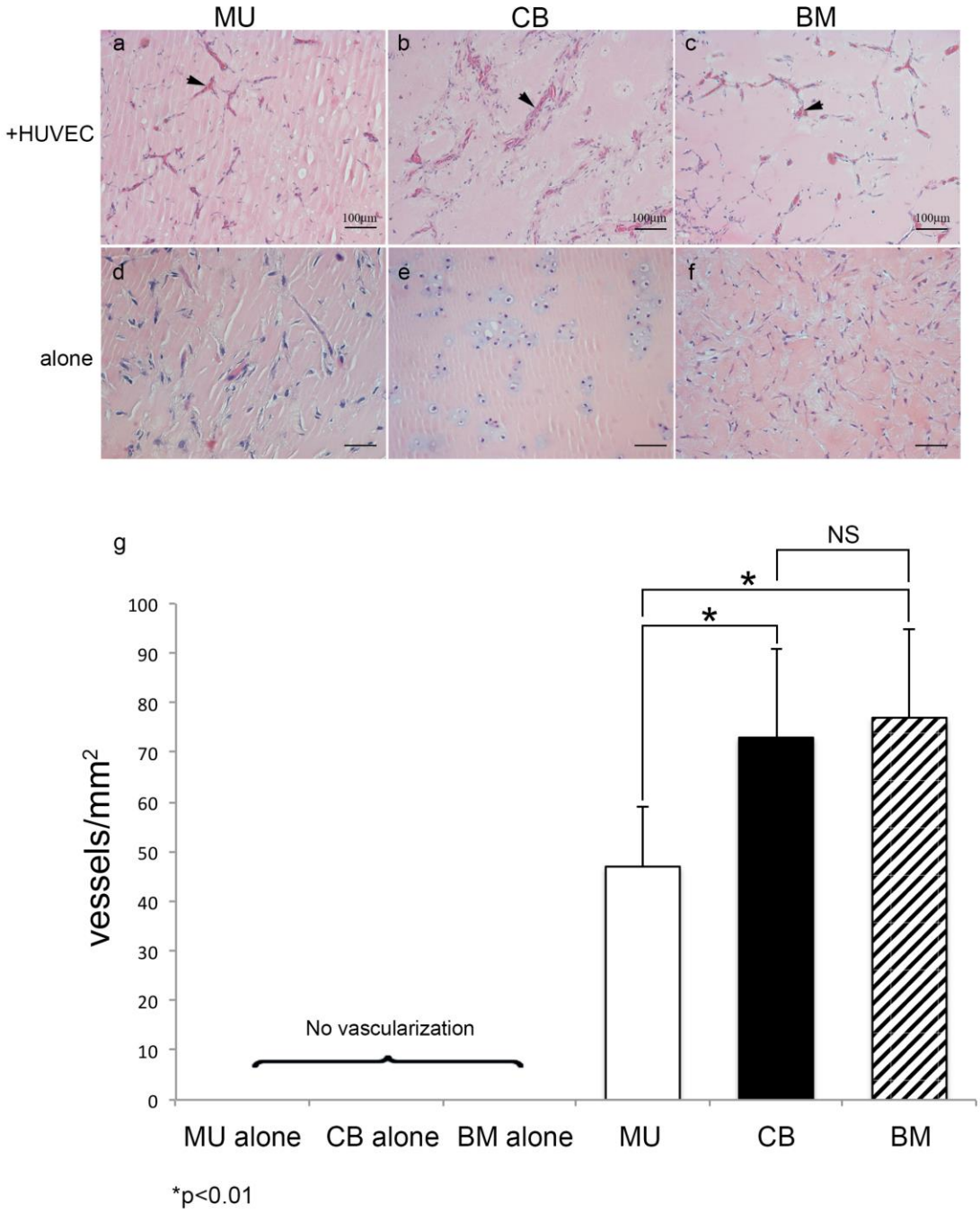
Supplemental Figure 3. Self-renewal of MU CD146^{+} cells.



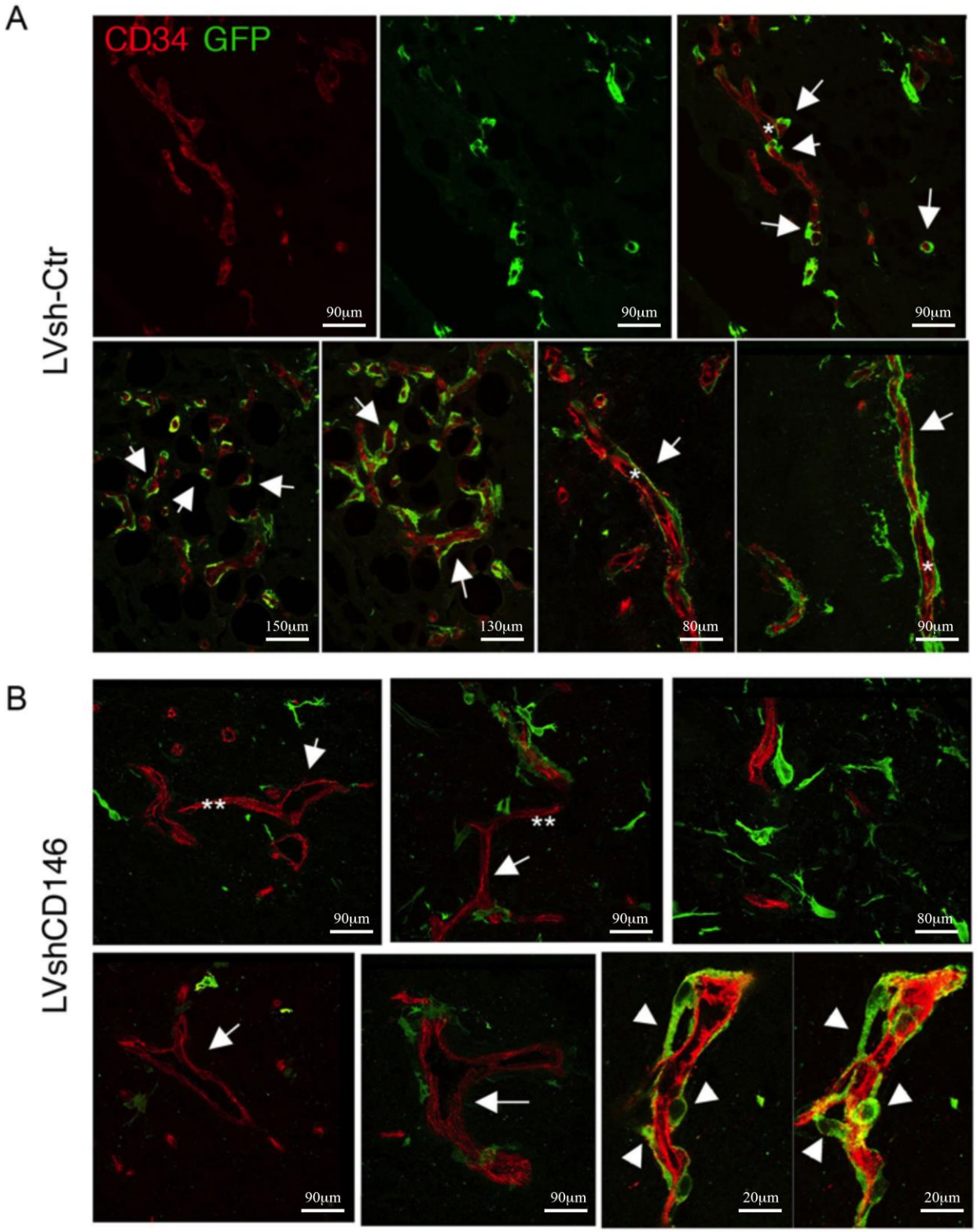
Supplemental Figure 4. In MU cells, expression of CD146, CD56 and CD34 by FACS fluorescent, immunohistochemistry and immunocytochemistry, and colony forming efficiency.



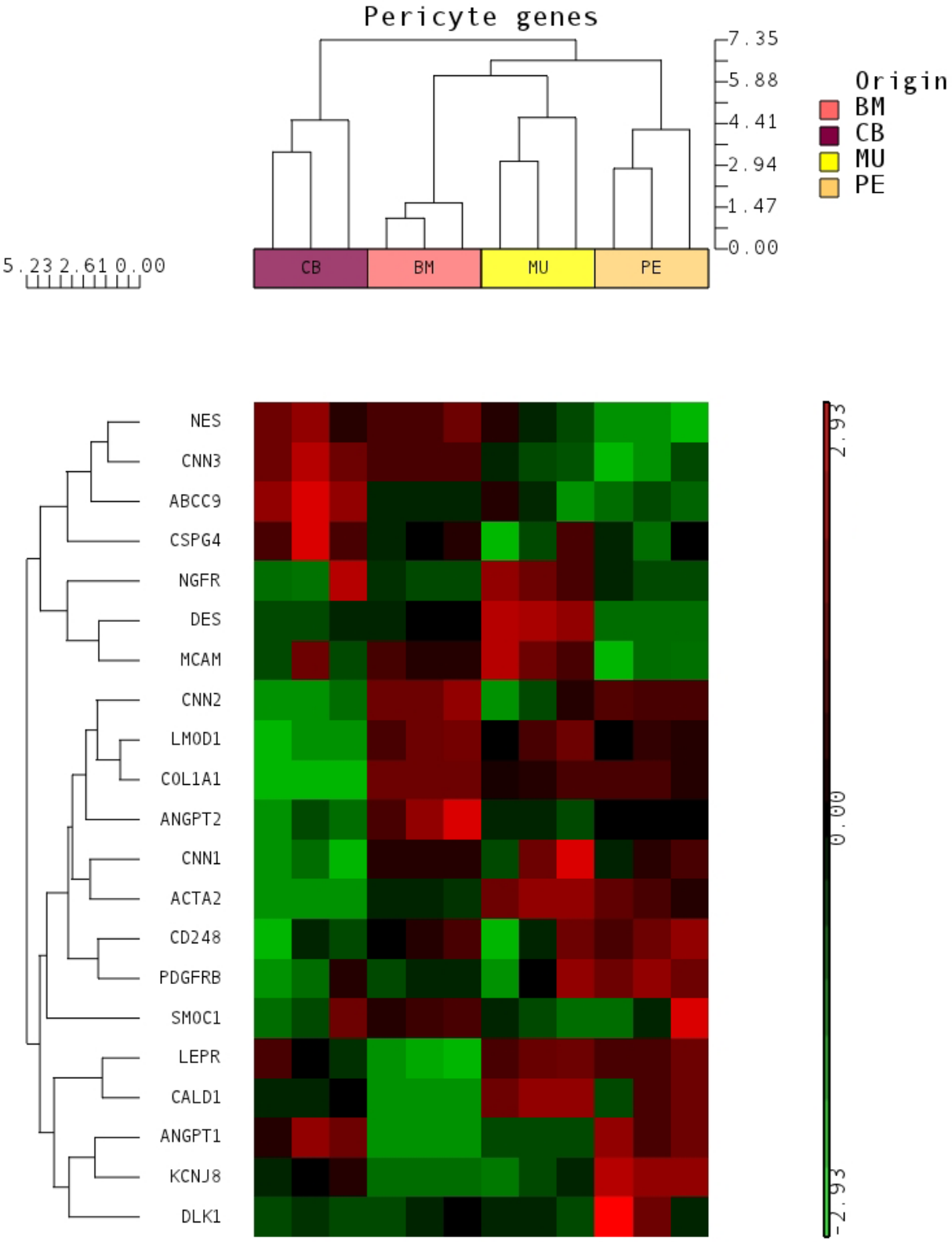
Supplemental Figure 5. Formation of vascular networks by "MSCs" *in vivo*.



Supplemental Figure 6. Effect of knockdown of CD146 in BMSCs in vessel formation with HUVECs in an *in vivo* transplantation assay.



Supplemental Figure 7. Comparison of pericyte-related genes expressed by "MSCs."



Supplemental Table 5. Quantification of satellite, mural cells and regenerative efficiency of CD146$^+$/CD34$^-$ cells *in vivo*

A. Quantification of satellite, mural* cells *of CD146$^+$/CD34$^-$ cells in SCID beige/ctx

Donor cell types huCD146$^+$/CD34$^-$	*Strain of mice*	*delivery*	*Number of injected cells*	*days after injection*	*% positive cells (mean±dev st)*
huCD56 (N-CAM)	SCID beige	i.m.	$1x10^6$	40	8.8 ± 2
huCD146 (M-CAM)	SCID beige	i.m	$1x10^6$	40	0.03 ± 0.01
huLamin A/C	SCID beige	i.m.	$1x10^6$	40	9.2 ± 2.7

B. Quantification of satellite, mural cells of CD146$^+$/CD34$^-$ cells in SCID/mdx

Donor cell types huCD146$^+$/CD34$^-$	*Strain of mice*	*delivery*	*Number of injected cells*	*days after injection*	*% positive cells (mean±dev st)*
huCD56 (N-CAM)	SCID/mdx	i.a.	$2x(5x10^5)$	15	2.6 ± 1.8
huLamin A/C	SCID/mdx	i.a.	$2x(5x10^5)$	15	3 ± 1.4

C. Regenerative efficiency of CD146$^{+/}$CD34$^-$ cells in vivo

Donor cell types huCD146$^+$/CD34$^-$	*Strain of mice*	*delivery*	*Number of injected cells*	*days after injection*	*N° positive fibers per muscle (mean±dev st)*
huSpectrin	SCID beige	i.m.	$1x10^6$	40	406 ± 47
huDystrophin 2	SCID/mdx	i.a.	$2x(5x10^5)$	15	543 ± 51.8
huDystrophin 3	SCID beige	i.m	$1x10^6$	40	503 ± 28.5

i.m., single intra-muscolar injection into tibialis anterior; ctx, cardiotoxin.
i.a., two consecutive intra-femoral artery injections.
i.m., single intra-muscular injection into *tibialis anterior*; i.a., two consecutive intra-femoral artery injections.
In all injected animals, only the *tibialis anterior* was analysed.

Data are the average of at least two independent experiments.

Supplemental Table 6. CFE assays for unsorted and sorted muscle cells.

A. CFE assay for unsorted, CD146^{+} and CD146^{-} muscle cells

Cells plated	*Cell density (cells/cm^2)*	*CFU-F*		
		Unsorted	CD146^{+}	CD146^{-}
1x10^2	1.6	2 ± 1.7	24 ± 21	0
2x10^2	3.3	ND	82 ± 2	0
1x10^3	16	10.3 ± 0.6	confluent	0
1x10^4	160	90 ± 20.5	confluent	0
1x10^5	1600	293 ± 47.4	ND	0

B. CFE assay for sorted CD146$^{+/-}$ and CD56$^{+/-}$ muscle cells

Cells plated	*Cell density (cells/cm^2)*	*CFU-F*		
		CD146^{+}/56^{-}	CD146^{-}/56^{+}	CD146^{-}/56^{-}
1x10^2	1.6	41.7 ± 4	0	ND
2x10^2	3.3	83.5 ± 8	0	ND
3x10^2	5	117.3 ± 0.4	0	ND
5x10^2	8.3	195.5 ± 0.7	0	0
1x10^3	16	ND	ND	0
1x10^4	160	ND	ND	0
1x10^5	1600	ND	ND	1 ± 1

C. CFE assay for sorted CD146$^{+/-}$ and CD34$^{+/-}$ muscle cells

Cells plated	*Cell density (cells/cm^2)*	*CFU-F*			
		CD146^{+}/34^{-}	CD146^{+}/34^{+}	CD146^{-}/34^{-}	CD146^{-}/34^{+}
1x10^2	1.6	16.5 ± 6.4	4 ± 1.5	0	0
3x10^2	5	31.5 ± 2.1	12 ± 4.2	0	0
5x10^2	8.3	52.5 ± 3.5	14 ± 1.4	0	0
1x10^3	16	ND	ND	0	0
1x10^4	160	ND	ND	0	0
1x10^5	1600	ND	ND	0	1 ± 1

D. CFE assay for sorted CD146^{+}/ALP$^{+/-}$ and CD146^{-}/ALP^{+} muscle cells

Cells plated	*Cell density (cells/cm^2)*	*CFU-F*	
		CD146^{+}/ALP$^{+/-}$	CD146^{-}/ALP^{+}
5	0.083	2 ± 0.7	0
50	0.83	21 ± 0.7	0
1x10^2	1.6	39.8 ± 0.4	0
2x10^2	3.3	79.5 ± 0.7	0
3x10^2	5	ND	8.2 ± 8.8
1x10^3	16	ND	28 ± 28

CFU-F, Colony Forming Unit-Fibroblastic; ND, not determined. Data are expressed as mean ± SEM. Data are from 2 independent experiments, each one done in triplicate.

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SHORT REPORT

Molecular profile of clonal strains of human skeletal stem/progenitor cells with different potencies☆

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Abstract
Bone marrow stromal cells (BMSCs, also known as bone marrow-derived mesenchymal stem cells) are fibroblastic reticular cells, a subset of which is composed of multipotent skeletal stem cells (SSCs). SSCs/BMSCs are able to recreate a bone/marrow organ in vivo. To determine differences between clonogenic multipotent SSCs and similarly clonogenic but non-multipotent BMSCs, we established single colony-derived strains (SCDSs, initiated by individual Colony Forming Unit-Fibroblasts) and determined their differentiation capacity by vivo transplantation. In this series of human SCDSs (N = 24), 20.8% formed fibrous tissue (F), 66.7% formed bone (B), and 12.5% formed a bone/marrow organ, and thus were multipotent (M). RNA isolated from 12 SCDSs just prior to transplantation was analyzed by microarray. Although highly similar, there was variability from one SCDS to another, and SCDSs did not strictly segregate into the three functional groups (F, B or M) by unsupervised hierarchical clustering. We then compared 3 F-SCDSs to 3 M-SCDSs that did segregate. Genes associated with skeletogenesis, osteoblastogeneis, hematopoiesis, and extracellular matrix were over-represented in M-SCDSs compared with F-SCDSs. These results highlight the heterogeneity of SSCs/BMSCs, even between functionally similar SCDSs, but also indicate that differences can be detected that may shed light on the character of the SSC.

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Introduction

Bone marrow (BM) stromal cells (BMSCs, also known as bone marrow-derived mesenchymal stem cells) were first identified and characterized by Friedenstein and Owen as a rapidly adherent, fibroblastic population of cells that contain a subset of multipotent stem cells (reviewed in Owen and Friedenstein, 1988). These cells are capable of recreating the hematopoietic microenvironment when transplanted in vivo (Friedenstein et al., 1974) by generating a bone/marrow organ. These ectopic ossicles have been consistently found to be composed of bone, hematopoiesis-supporting stroma, marrow adipocytes of donor origin, and hematopoiesis of recipient origin (Balakumaran et al., 2010; Dieudonne et al., 1998; Krebsbach et al., 1997; Kuznetsov et al., 1997; Sacchetti et al., 2007). Subsequent studies have shown that these skeletal stem cells (SSCs, Bianco and Robey, 2004) are self-renewing, sub-endothelial cells that line BM sinusoids (pericytes) and send out processes that intercalate into areas of hematopoiesis (Sacchetti et al., 2007). Consequently, SSCs are hypothesized to be important participants in the hematopoietic stem cell (HSC) niche (Mendez-Ferrer et al., 2010; Sacchetti et al., 2007).

Much work has been done on studying the biological activities of BMSCs in vitro. While in vitro assays are valuable tools to address specific questions, they are not well suited for studying the biological activities of SSCs directly, due to the fact that the latter represent only a subset of cells within the BMSC population. Furthermore, there is no single marker or set of markers that can efficiently separate SSCs from non-multipotent BMSCs (Bianco et al., 2008), and even if there were, ex vivo expansion would result again in a mixture of stem cells and more committed cells due to the kinetics of cell division (reviewed in Neumuller and Knoblich, 2009). If one assumes that stem cell division is strictly asymmetrical (one cell remaining a stem cell, the other a more committed cell), the stem cell subset would rapidly be diluted by transiently amplifying cells that are not stem cells (Kuznetsov et al., 2004). In addition, while SSCs are clearly a component of the HSC niche, current culture conditions required for support of human HSCs in vitro are not optimal (Lymperi et al., 2010). For these reasons, in vivo transplantation is the gold standard by which to characterize the differentiation capacity of a clonal BMSC population, in particular with regard to the formation of hematopoiesis-supportive stroma, a defining feature of SSCs (Bianco, 2011). Furthermore, only a subset of freshly isolated BMSCs are capable of density-independent growth [Colony Forming Unit-Fibroblasts (CFU-Fs)], and the resulting clones are heterogeneous in their in vitro differentiation potential (Muraglia et al., 2000; Pittenger et al., 1999; Russell et al., 2010 as examples), and their ability to recreate a bone/marrow organ in vivo (Friedenstein, 1980; Gronthos et al., 2003; Kuznetsov et al., 1997; Sacchetti et al., 2007). In these studies, 10–20% of the single colony-derived strains (SCDSs, initiated by individual CFU-Fs) formed a bone/marrow organ, while the remainder formed only bone (35–45%) or fibrous tissue (35–55%).

Currently, the molecular profile of subsets of SSCs/BMSCs with varying differentiation potentials is largely undefined. Larsen et al. previously established transcription profiles that distinguish between immortalized clones with and without the ability to form bone in vivo (Larsen et al., 2010). Clones that formed bone had increased expression of extracellular matrix genes, and those that did not form bone expressed immune response-related genes. Here we present data from primary unmodified SCDSs. We first established the functionality of human SCDSs by in vivo transplantation, and then compared the molecular signature of SCDSs that regenerated a complete bone/marrow organ with those that formed only fibrous tissue.

Materials and methods

Generation of BM single cell suspensions

A suspension of BM nucleated cells (BMNCs) was prepared from human trabecular bone from surgical waste of a single donor (female, 43 years-old) according to NIH guidelines as previously described (reviewed in Robey et al., 2014). Briefly, BM was gently scraped from bone fragments into growth medium [α-MEM, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin (all from Invitrogen), and 20% lot-selected, non-heat inactivated fetal bovine serum (HyClone)], and the fragments were washed extensively to remove marrow. After pelleting by centrifugation, bone marrow nucleated cells (BMNCs) were resuspended in growth medium, and passed through a 16 gauge needle, and subsequently through a 70 μm cell strainer (Becton Dickinson) to remove aggregates.

Generation of SCDSs and non-clonal BMSCs

SCDSs were prepared as previously described (Robey et al., 2014). BMNCs were plated at low density (2×10^3 nucleated cells/cm^2) into 150 mm^2 tissue culture dishes (Becton Dickinson), and cultured in growth medium at 37 °C for 14 d without any media replacements. After 14 d, single colonies with >50 cells were randomly selected and individually isolated. Only colonies with a round morphology and obvious separation from surrounding colonies were chosen. The colonies were isolated by attaching a cloning cylinder (Bel-Art Products) to the dish using sterile vacuum grease (Baxter Healthcare Corp.). Cells were detached with 0.05% Trypsin/EDTA (Invitrogen), and transferred to 1 well of a 6-well dish (Becton Dickinson) with growth medium. Once the cells approached confluency, they were passaged consecutively into one 75 cm^2 flask (Becton Dickinson), and then into two 75 cm^2 flasks. Once these flasks became confluent, the cells were detached and used for RNA isolation and in vivo transplantation. In some cases, SCDSs generated previously and cryopreserved from another donor, were used in experiments described below. Non-clonal BMSC cultures were established by plating BMNCs at a density of 5×10^6–5×10^7 cells/75 cm^2 tissue culture flask in growth medium as previously described (Robey et al., 2014).

RNA isolation

RNA was isolated from at least 5×10^5 cells from each SCDS or non-clonal BMSC cultures with TRIzol (Invitrogen), and further purified by using a combination of chloroform phase

separation and RNeasy Mini Kits (Qiagen) according to the manufacturer's protocol.

In vivo transplantation of SCDSs

2×10^6 cells from each SCDS were suspended in growth medium and incubated at 37 °C on a rotator with 40 mg of sterile hydroxyapatite/tricalcium phosphate particles (HA/TCP, Zimmer). After 90 min, the particles and cells were collected by brief centrifugation and transplanted subcutaneously into the flank of an immunodeficient mouse (NIH-*Lyst*$^{bg\text{-}J}$*Foxn1*^{nu}*Btk*^{xid}, Charles River). After 8 wks, the mice were euthanized and the transplants harvested (Krebsbach et al., 1997).

Histological scoring of in vivo transplants

Each transplant was fixed in 4% paraformaldehyde for 3 d, and decalcified in 0.25 M EDTA. Decalcification was confirmed by X-ray (Faxitron, 30 V for 40 s using Kodak PPL film). After decalcification, the transplants were embedded in paraffin and sectioned (5 μm). Each transplant was sectioned such that a section was taken every 100 μm to generate at least five sections, in order to ensure procurement of a representative sample through the entire thickness of each transplant. Sections were stained with H&E and examined microscopically. Each section was given a separate semi-quantitative score ranging from 0 to 4 for both bone and hematopoiesis formation by two independent, trained observers (see Mankani et al., 2004). Bone and hematopoiesis were scored independently of each other; for example, a section with exuberant bone formation but less abundant hematopoiesis could receive a score of 4 for bone formation, but only 1 for hematopoiesis.

Gene expression microarray analysis

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. RNA was quantified and 5 μg was processed for microarray analysis (LMT, NCI, Frederick, MD). RNA was reverse transcribed to form cDNA, and hybridized to Affymetrix GeneChip Human Genome U133 Plus 2.0 arrays, composed of more than 54,000 probe sets and 1,300,000 distinct oligonucleotide features that analyze the expression level of over 47,000 transcripts and variants, including 38,500 well-characterized human genes. Three independent replicates for each of the experimental conditions were carried out and analyzed to control for intra-sample variation. Comparative analyses of expressed genes that were over/under-represented by >2-fold were carried out using the GeneSpring software. Signal intensity values were normalized using RMA summarization and baseline transformation to median of all samples was performed. Entities were filtered based on their signal intensity values. A total of 45,371 out of 54,675 entities passed the test where 1 out of 6 samples have signal intensity values between 20 and 100 percentiles. Hierarchical clustering was performed on filtered signal intensity (>20.0), non-averaged, fold change > 2. Gene ontology analysis was done using fold change > 2, and a p-value cutoff of 0.1, as a p-value cutoff 0.05 resulted in no significant GO groups. A fold change analysis (>10 fold) was performed to generate a list of top genes over/under-represented between groups. Statistical analysis was performed using the Student's *t*-test (fold change ≤ 2, corrected p-value ≤ 0.05).

qRT-PCR analysis

RNA was extracted from cells that remained after generation of in vivo transplants and microarray analysis (SCDS-61, SCDS-11, SCDS-99, SCDS-107), from SCDSs isolated from another donor, and from non-clonal BMSCs. RNA (1.0 μg) was used for cDNA synthesis using the iScript cDNA Synthesis Kit (Bio-Rad, #170-8891). Quantitative RT-PCR (qPCR) was performed using a CFX-96 Real Time System paired with a C1000 Thermal Cycler (Bio-Rad, Hercules, CA). qPCR reactions were set up using iQ SYBR Green Supermix (Bio-Rad #170-8882) according to the kit's instructions. Primers were designed using Beacon Designer 6 software (Premier Biosoft International, Palo Alto, CA): hSFRP2 (NM_003013) — F: AGGACAA CGACCTTTGCATC, R: CAGGCTTCACATACCTTTGGA; hCNN1 (NM_001299.4) — F: ACTTCATGGACGGCCTCA, R: TGGGTTG ACTCATTGATCTTCTT; RPL13a (NM_012423) — F: GGAGAA GAGGAAAGAGAAAGC, R: GGCAACAATGGAGGAAGG; GAPDH (NM_002046.3) — F: TGCACCACCAACTGCTTAGC, R: GGCAT GGACTGTGGTCATGAG. qPCR results, expressed as critical threshold (C_T) values, were normalized to the levels of RPL13a or GAPDH, generating ΔCT values; levels of relative expression were calculated as $2^{-\Delta CT}$.

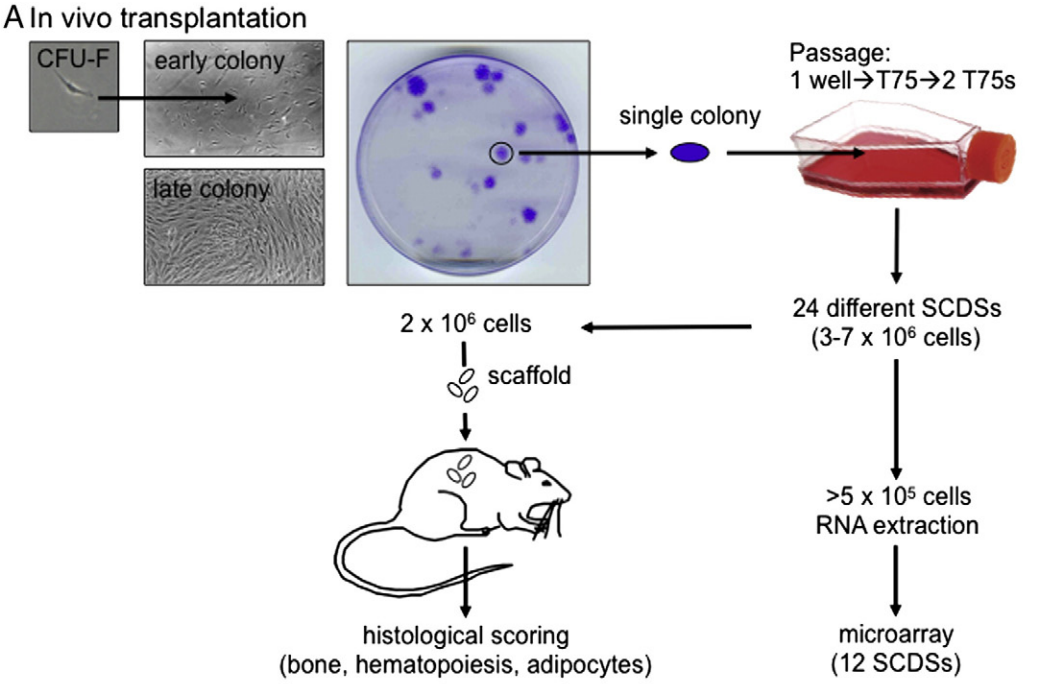
Results

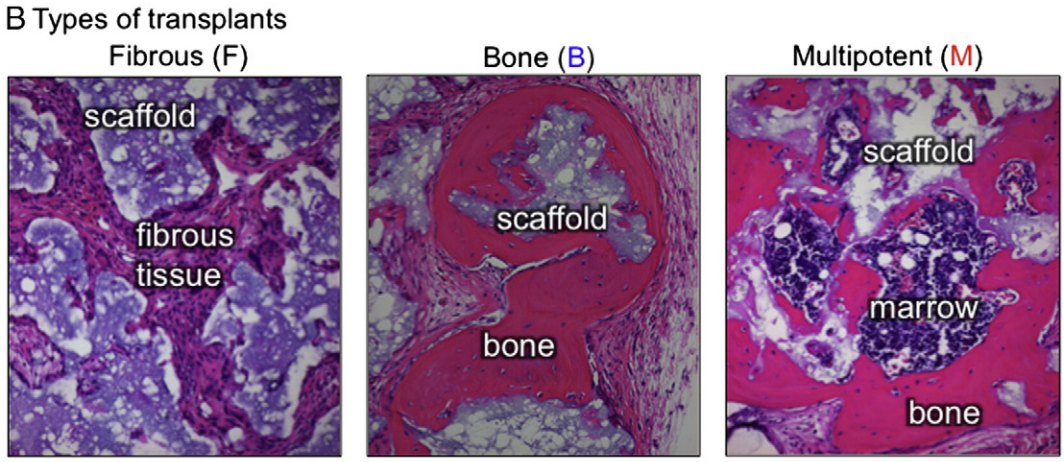
Generation and transplantation of SCDSs

SCDSs of hBMSCs were expanded under basal conditions, and their in vivo differentiation potential was assayed according to the scheme represented in Fig. 1A. Of the 114 hBMSC colonies originally selected, ~50% ceased to proliferate. Of those that continued to proliferate, 24 were randomly selected, transplanted, and harvested after 8 wks. Of note, no adipocytic differentiation was noted in any of the SCDSs. Between ~3 and 7×10^6 cells were available at the time of transplant.

Histological analysis and scoring of SCDS transplants

Sections of transplants were stained with H&E, examined histologically, and given an independent score ranging from 0 to 4 for both bone and hematopoiesis (Mankani et al., 2004). Those clonal strains that formed only fibrous tissue were considered to be devoid of SSCs, and were termed fibrous (F), those that formed bone without supporting hematopoiesis were considered to be unipotent (B), and those that formed bone and supported formation of marrow (stroma and adipocytes of donor origin, hematopoiesis of recipient origin) were considered to be multipotent (M) (Fig. 1B). Of the 24 SCDSs transplanted, 5 (20.8%) formed fibrous tissue (F), 16 (66.7%) formed bone (B), with scores between 1 and 3, and 3 (12.5%) were multipotent (M) (Fig. 1C). These results are consistent with what has been reported previously. Notably, only M transplants were found to have adipocytes; adipocytes were not found in F or B transplants.





C Scoring

	Fibrous	Bone Only				Multipotent		
Transplant Score - Scale of 0-4 (Bone Score/HP Score)	0/0	1/0	2/0	3/0	4/0	3/1	3/2	4/2
Number of Clones	5	5	7	3	1	1	1	1
	20.8%	66.7%				12.5%		

Figure 1 A) Experimental design for generation of single colony-derived strains (SCDSs), assessment of functionality by in vivo transplantation and profiling by microarray analysis. B) Types of transplant. Twenty-four SCDSs were transplanted in vivo with hydroxyapatite/tricalcium phosphate ceramic particles as a scaffold, and then scored on a scale of 0–4 for the presence of bone and hematopoiesis. The SCDSs were categorized as generating fibrous tissue (no bone, no support of hematopoiesis) (F), bone-forming only (B), and as multipotent (M) based on the formation of bone and support of hematopoiesis. C) The scores of individual transplants were categorized, and the percentages of each type (F, B or M) are indicated.

Analysis of SCDS gene expression using microarray

RNA from a total of 12 SCDSs (5 F, 4 B, and 3 M) was analyzed by microarray. All 12 SCDSs were allowed to undergo unsupervised hierarchical clustering based on total gene expression (Fig. 2A). Based on the heat map, the SCDSs were highly similar to one another, but there also was a fair amount of variability. In addition, the SCDSs did not strictly segregate into the three distinct functional groups (F, B and M). The 3 M-SCDSs clustered tightly together, but F-SCDSs clustered into 2 different groups.

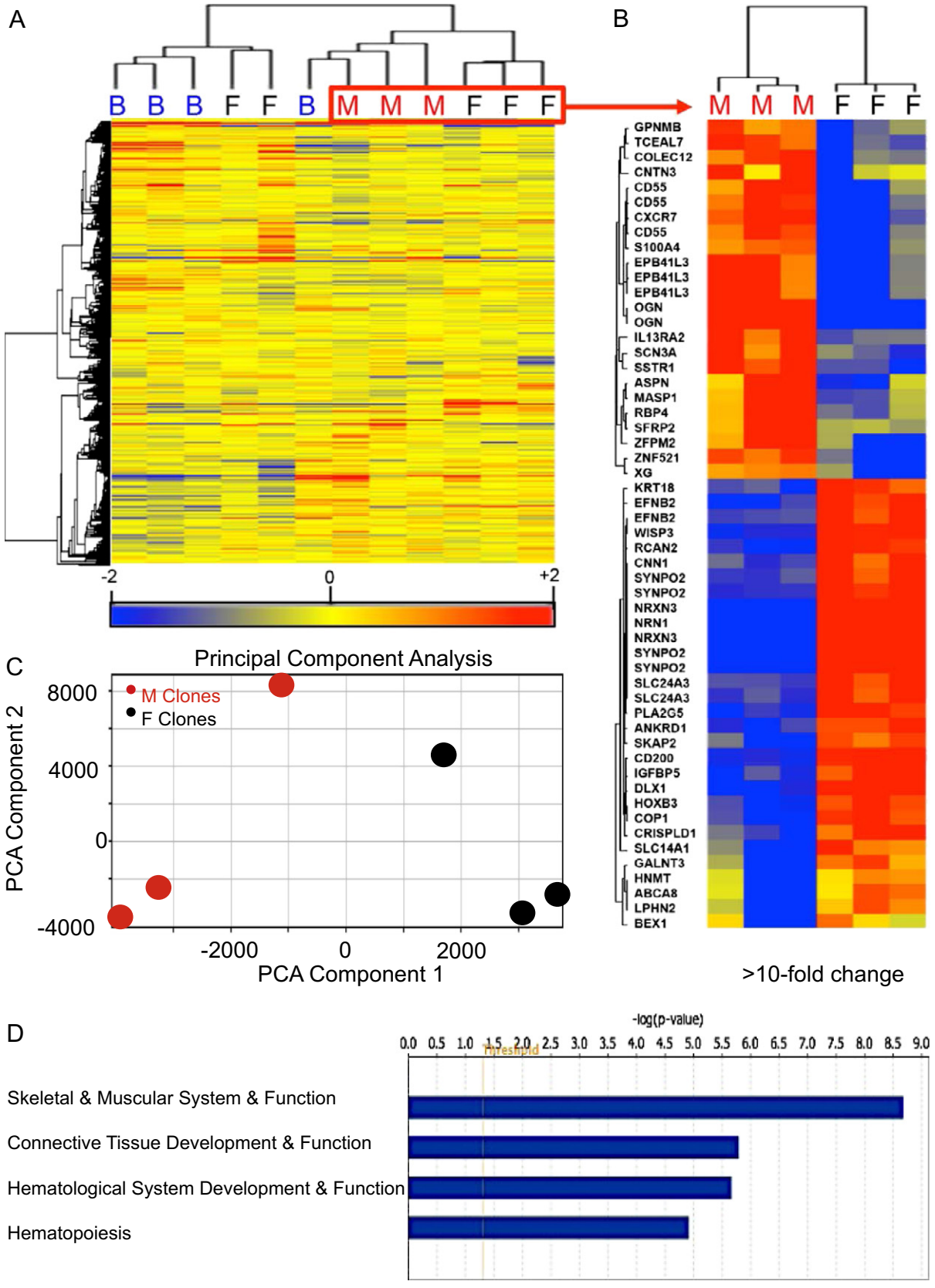


Figure 2 Microarray analysis of 12 SCDSs with known functionality based on the results of in vivo transplantation (5 F, 4 B, and 3 M). A) Unsupervised hierarchical clustering and the heat map generated by microarray. B) Unsupervised hierarchical clustering and heat map of 3 M-SCDSs (M-61, M-11, M-2), and the three F-SCDSs (F-99, F-017, F-109) that were used for further analysis of the patterns of gene expression. C) Principle component analysis of the 3 M-SCDSs (red circles) and the 3 F-SCDSs (black circles). D) GO groups highly represented in M-SCDSs compared to F-SCDSs.

Table 1 Genes differentially expressed by M-SCDSs relative to F-SCDSs.

Gene name	Symbol	Fold change	Direction
A. Genes involved in skeletogenesis			
Mesenchyme homeobox 2	*MEOX2*	4.77	Up
GNAS complex locus	*GNAS*	2.17	Up
Eyes absent homolog 1	*EYA1*	2.74	Up
Paired related homeobox 1	*PRRX1*	2.03	Up
Calponin 1, basic, smooth muscle	*CNN1*	11.27	Down
B. Genes involved in osteoblast differentiation			
Bone morphogenetic protein 2	*BMP2*	2.65	Up
Bone morphogenetic protein 4	*BMP4*	2.34	Up
Secreted frizzled-related protein 2	*SFRP2*	33.14	Up
Msh homeobox 2	*MSX2*	2.41	Up
Insulin-like growth factor 1	*IGF1*	6.30	Up
Meningioma 1	*MN1*	2.90	Up
Wnt1-inducible-signaling pathway protein 1	*WISP1*	2.83	Up
C. Genes involved in hematopoiesis			
Bone morphogenetic protein 4	*BMP4*	2.34	Up
Intercellular adhesion molecule 1 (CD54)	*ICAM1*	2.01	Up
Interleukin 8	*IL8*	2.86	Up
Chemokine (C-X-C motif) ligand 1	*CXCL1*	4.27	Up
D. Small leucine rich repeat proteoglycans			
Decorin	*DCN*	2.33	Up
Osteoglycin	*OGN*	35.30	Up
Osteomodulin	*OMD*	9.93	Up
Asporin	*ASPN*	26.43	Up

2 F-SCDSs clustered with 3 B-SCDS, while 3 others clustered with each other, and along with M-SCDSs. One B-SCDS clustered separately with the M-SCDSs.

Because of the heterogeneity noted, the 3 F-SCDSs that clustered together and the 3 M-SCDSs were chosen for further analysis, as it would allow us to determine the differences in gene expression between two extremes of the differentiation spectrum (multipotent vs. fibrous). Heat maps showing all genes (Fig. 2A), and only the genes that were at least 10-fold differentially expressed between the M-SCDSs and F-SCDSs (Fig. 2B), revealed that there were considerable differences between SCDSs with the same in vivo differentiation potential (see also Supplementary Table 1 for genes over-and under-represented at >10-fold and >5-fold, GEO accession number: GSE647890). This is further reflected in principle component analysis of these SCDSs. While the M and F groups separate, those within each group are not tightly clustered (Fig. 2C).

Ingenuity pathway analysis (IPA) of higher-level functions

IPA was used to determine which biological and molecular functions were significantly associated with the genes that were different between the M-SCDSs and F-SCDSs by at least two-fold (Fig. 2D). Among the functional categories identified were those associated with Skeletal and Muscular System Development and Function, Connective Tissue Development and Function, (both pointing to the inherent osteogenic nature of M-SCDSs), Hematological System Development and Function, and Hematopoiesis, again highlighting the participation of M-SCDSs in the HSC niche.

Differential gene expression

Examination of genes in the IPA categories that were significantly over-represented in M-SCDSs by >2-fold revealed genes involved in skeletogenesis (Table 1A — *MEOX2*, *GNAS*, *EYA1*, *PRRX1*) and osteoblast differentiation (Table 1B — *BMP2*, *BMP4*, *SFRP2*, *MSX2*, *IGF1*, *MN1*, *WISP1*). Interestingly, *WISP3*, a close relative of *WISP1* was highly under-represented (Fig. 2B). Hematopoiesis-related genes were also over-expressed (Table 1C — *BMP2*, *ICAM1*, *IL8*, *CXCL1*), including CXCR7 (Fig. 2B), which binds to CXCL11 and CXCL12, although its role in hematopoiesis is not yet known. In addition, 4 members of the small leucine-rich repeat proteoglycan family (SLRP) were over-represented (Table 1D — *DCN*, *OGN*, *OMD*, *ASPN*). Based on our analyses, *SFRP2* was the most significantly over-represented gene, while *CNN1* was the most significantly under-represented (Table 1A). More information concerning the role of these genes in skeletogenesis, osteogenesis, hematopoiesis and extracellular matrix function can be found in the Supplementary Information.

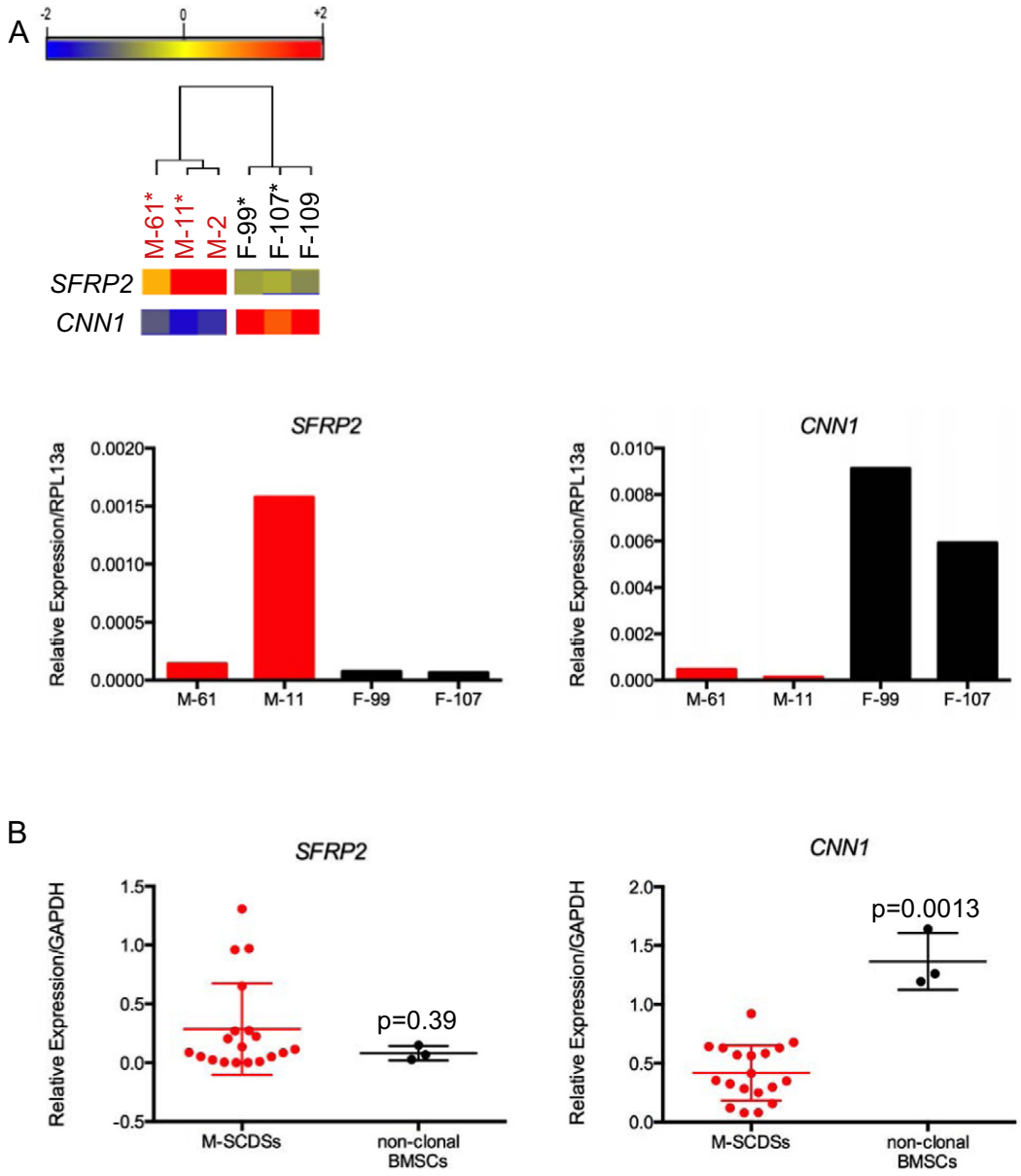


Figure 3 Evaluation of *SFRP2* and *CNN1* expression in M-SCDSs, F-SCDSs and non-clonal BMSCs by qRT-PCR. A) RNA was extracted from cells remaining after in vivo transplantation and microarray analysis (M-SCDS-61, M-SCDS-11, F-SCDS-99 and F-SCDS-107 indicated by asterisks); no more cells were available for any of the other SCDS strains shown in Fig. 2A). *SFRP2* (highly over-represented, Fig. 2B), was variably expressed in the two remaining M-SCDSs, coinciding with what was found in the heat map (Fig. 2B). On the other hand, expression of *CNN1* (highly under-represented, Fig. 2B), was under-expressed in both M-SCDSs and highly expressed in both F-SCDSs, consistent with what was found in the heat map (Fig. 2B). B) The ability of *SFRP2* and *CNN1* to distinguish between M-SCDSs from another donor compared to non-clonal BMSCs was examined. As with the original M-SCDSs (Fig. 3A), *SFRP2* was highly variable in the series of M-SCDSs from another donor, although several M-SCDSs expressed high levels. *CNN1* was clearly under-expressed in M-SCDSs compared with non-clonal BMSCs.

Determination of *SFRP2* and *CNN1* expression by qRT-PCR

RNA was extracted from all cells that were remaining (M-SCDS-61, M-SCDS-11, F-SCDS-99, F-SCDS-107, Fig. 3A), and analyzed for expression of *SFRP2* and *CNN1*, which were highly over-represented and under-represented in M-SCDSs, respectively. As shown in Fig. 3A, the expression of *SFRP2* was variable between the two M-SCDSs, with one having much higher expression than the other, reflective of what was observed on the heat map. On the other hand, *CNN1* was more consist, with a greatly reduced level of expression in M-SCDSs vs. F-SCDSs.

We next sought to determine whether the markers identified in the current series of SCDSs would be detected in the same pattern in SCDSs from a different donor. A number of M-SCDSs from another donor had been previously

cryopreserved; however, no F-SCDSs grown under the same conditions were available. For that reason, we compared the levels of *SFRP2* and *CNN1* expression in the M-SCDSs from the second donor to three different non-clonal populations of BMSCs, which are a mixture of cells at various stages of commitment. While there was a trend for higher expression of *SFRP2* in M-SCDSs (4/19 had much higher, and 4/19 had slightly higher expression), the difference compared with non-clonal BMSCs was not statistically different (Fig. 3B). This variability is similar to what was observed in the original series of M-SCDSs (Fig. 3A). Expression of *CNN1* by the M-SCDSs was significantly lower than in non-clonal BMSCs (Fig. 3B).

Discussion

Here we establish a molecular signature for unmodified SCDSs that were initiated by individual multipotent SSCs in comparison with SCDSs that were initiated by cells that were not multipotent, based upon their differentiation capacity as determined by in vivo transplantation. Of note, all of our SCDSs were established by clonogenic cells (CFU-Fs), but only ~1 out of 5 was in fact multipotent, as has been reported previously by us, and others (Friedenstein, 1980; Gronthos et al., 2003; Kuznetsov et al., 1997; Sacchetti et al., 2007). This reinforces the notion that cultures of BMSCs should not be referred to as "stem cell" cultures (as is often the case), but as cultures in which of a subset composed of stem cells exist. Not even all CFU-Fs are stem cells, although their enumeration provides an approximation of the number of stem cells within a freshly isolated single cell suspension of BM (Bianco et al., 2008).

The results show that the molecular profiles of SDSCs were very similar to one another, but no two were alike, supporting the view that BM stromal CFU-Fs are heterogeneous. It has long been noted that upon plating of cells at clonal densities, there are differences in the size and growth habit (monolayer or multilayering) of colonies. Previous studies (e.g., Satomura et al., 1998) showed a positive correlation between rate of proliferation and multipotency of murine SCDS based on in vivo transplantation. In our current series, M-SCDSs appeared to proliferate slightly faster than F-SCDSs based on the number of days it took to reach the final harvest and the total number of cells generated, but this was not statistically significant (data not shown). Furthermore, colonies are composed of cells of different shapes and sizes, ranging from extended fibroblastic cells to large flat cells (Digirolamo et al., 1999; Owen and Friedenstein, 1988; Satomura et al., 1998). However, the morphological nature of the colony was not predictive of the outcome of in vivo transplantation assays (Satomura et al., 1998). When colonies are allowed to spontaneously differentiate upon prolonged culture, varying percentages of osteogenic, adipogenic or non-differentiated colonies arise (Owen and Friedenstein, 1988). This may be indicative of commitment of a particular CFU-F to one of the stromal cell phenotypes, as a reflection of the influences exerted on that CFU-F during embryonic growth, and post-natal development and homeostasis. With passage, the size and shape of the cells become more uniform; however, heterogeneity still persists, based on the fact that not all cells retain the ability to form colonies upon re-plating at clonal density (Friedenstein, 1976). This is most likely due to the kinetics of SSC self-renewal that are not yet well understood in mammalian systems (Neumuller and Knoblich, 2009). Furthermore, the rate of proliferation of cells within an established colony (as would be harvested at 14 d) is not synchronized, with cells in the periphery migrating and proliferating at a faster rate than those that are more central (Friedenstein, 1990). These differences result in cells within the colony being in different phases of the cell cycle, which can impact on gene expression. For example, alkaline phosphatase is shed from the cell during the G2 + M phase, and is slowly regained during G1 and S phases (Fedarko et al., 1990).

Secondly, our study showed that SCDSs did not strictly segregate transcriptionally based on their differentiation potential as determined by in vivo transplantation. The basis for this is not clear, but may relate to a lack of knowledge concerning the stages of maturation of SSCs (pericytes) to more mature phenotypes (osteoblasts, adipocytes, stromal cells). Stages of osteogenic differentiation have been marked by use of mouse reporter lines that suggest that Runx2 is expressed in SSCs/BMSCs, and committed osteoprogenitors (Yoshida et al., 2002), Osterix is expressed in immature osteogenic cells (Maes et al., 2010), the Col1a1 2.3 kb promoter is active in more mature osteoblastic cells (Pavlin et al., 1992), and that Osteocalcin is expressed in very mature osteoblasts and osteocytes (Zhang et al., 2002). However, such staging for SSC/BMSC differentiation is not yet clear. Based on the hierarchical clustering (Fig. 2A), it can be speculated that a cell that initiated a B-SCDS that clustered with M-SCDSs represented a cell that was in transition from being multipotent to a committed osteogenic cell. Likewise, the individual cells that initiated the F-SCDSs that clustered with other B-SCDSs may have recently transitioned to a fibroblastic phenotype from an osteogenic phenotype. The fact that the F-SCDSs clustered into 2 distinct groups (one with B-SCDSs, the other with M-SCDS) suggests that while all of the F-SCDSs could not make bone in vivo, there may be at least two subsets of fibroblastic BMSCs. A plausible explanation for the fact that 3 F-SCDSs clustered with M-SCDSs may relate to the fact that committed osteogenic cells (B-SCDSs) have a quite different repertoire of expressed genes compared to those that do not exert an overt phenotype (M-SCDSs and F-SCDSs). Clearly, further investigation will be needed to establish the hierarchy of SSCs/BMSCs.

Despite the high degree of similarity between SCDSs and the lack of strict segregation by function, we did identify genes that were highly over-represented by comparing 3 M-SCDSs and 3 F-SCDSs (see Supplementary Information for the role of these differentially expressed genes in skeletogenesis, osteogenesis, hematopoiesis and extracellular matrix). While there are numerous reports on the molecular signature of "mesenchymal stem cells," most of these studies have focused on comparing profiles of "MSCs" from different tissues (e.g., Al-Nbaheen et al., 2013), or BMSCs after initiating differentiation (e.g., Delorme et al., 2009). One study (Papadimitropoulos et al., 2014) in which 3D culture conditions appeared to maintain stemness better that 2D conditions reported over-representation of IL8 and DNER, and under-representation of SYNPO2, NTN4 and LIMCH1 which were over- and under-represented by

>2-fold respectively (data not shown) in our M-SCDSs (with the exception of SYNPO2, which was >10-fold down regulated, see Fig. 2). Few studies have evaluated SCDSs for both their in vivo differentiation potential and their molecular signature. Larsen et al. (2010) reported that their immortalized bone-forming clone highly expressed extracellular matrix genes, including DCN, IL-8 and IFI27, all of which were expressed >2-fold higher in our M-SCDSs (Supplementary Table 1, and data not shown), but the status of hematopoiesis in transplants generated by this immortalized clone was not reported. In another study, clones isolated from different tissues, including BM, and profiled based on rate of proliferation and differentiation, identified *TWIST1* as highly over-represented (Menicanin et al., 2010), as it was in our M-SCDSs (>2-fold, data not shown).

The strengths of our studies are that we were able to detect differences in the molecular profiles of SCDSs derived from a single donor that were initiated by multipotent SSCs vs. BMSCs that were not. However, it is known that the growth rate, expression of markers and differentiation capacity are variable from donor to donor (Phinney et al., 1999). To address this issue, we used a series of M-SCDSs from another donor, and found that *SFRP2*, while highly expressed in some M-SCDSs, was not as robust in identifying M-SCDSs as decreased expression of *CNN1*. These data highlight the need to analyze a large series of SCDSs from more donors to identify reliable and predictive markers. Perhaps the more significant issue relates to the need to expand the cells ex vivo, which may exert proliferative stress that leads to shortened telomeres, DNA damage and changes in differentiation capacity, to obtain sufficient numbers of cells for establishment of their functionality by the in vivo transplantation assay and concomitant molecular profiling. As noted above, heterogeneity exists within colonies, thereby masking what may be profound differences between SSCs and cells that become more committed as the colony is established, and propagated. Analyzing freshly isolated single cells without ex vivo expansion, as recently performed on hematopoietic cells (Guo et al., 2013), would undoubtedly eliminate this issue. Nonetheless, our results begin to better describe the heterogeneous nature of SSCs/BMSCs that has been postulated, but not clearly defined at the molecular level. Future studies will attempt to adapt a single cell strategy, although it will not be possible to study what a given cell would do upon in vivo transplantation. The genes identified in our current study may help guide identification of a single cell profile representative of an SSC in this type of approach.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2015.02.005.

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COMMENT

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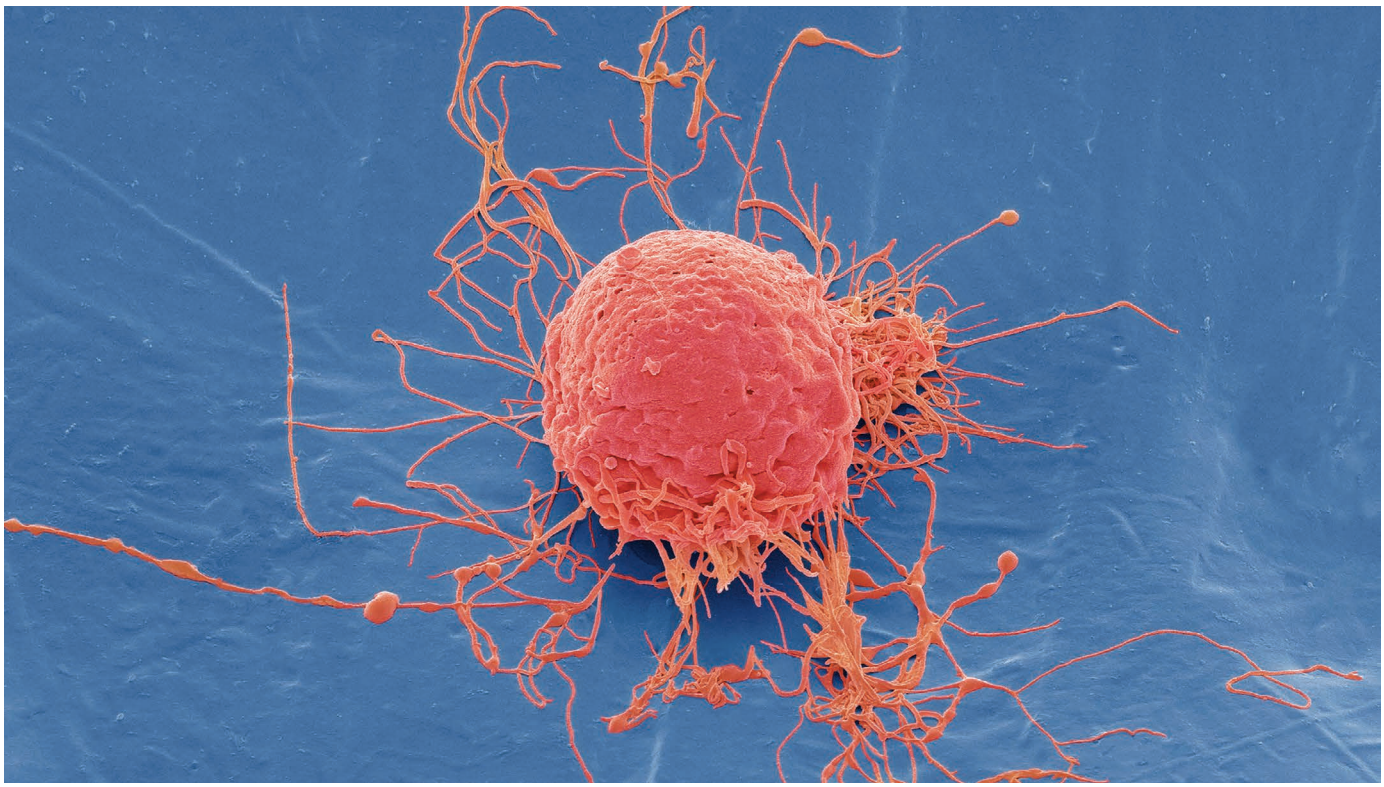
GENOMES Don't use genomics to excuse social inequality **p.461**

AUTHORSHIP Follow the film industry and list contributions instead **p.464**

CONFERENCES Do boring speakers talk for longer, or does it just feel that way? **p.464**

STEVE GSCHMEISSNER/SPL



Scanning electron micrograph of what is called a human mesenchymal stem cell, which some say can develop into bone, cartilage or fat cells.

Clear up this stem-cell mess

Confusion about mesenchymal stem cells is making it easier for people to sell unproven treatments, warn **Douglas Sipp**, **Pamela G. Robey** and **Leigh Turner**.

Various populations of cells in the adult human body have been the subject of controversy since the early 2000s. Contradictory findings about these haphazardly termed 'mesenchymal stem cells', including their origins, developmental potential, biological functions and possible therapeutic uses, have prompted biologists, clinicians and scientific societies to recommend that the term be revised or abandoned. Last year, even the author of the paper that first used the term mesenchymal stem cells (MSCs) called for a name change[1].

Tissue-specific stem cells, which have a limited ability to turn into other cell types, are the norm in most of the adult body. Several studies indicate that the variety of cells currently dropped into the MSC bucket will turn out to be various tissue-specific cell types, including stem cells[2].

Yet the name persists despite the evidence pointing to this, and almost two decades after questions about the validity of MSCs were first raised. A literature search indicates that, over the past 5 years, more than 3,000 research articles referring to MSCs have been published every year (see 'Tenacious term'). And several national regulatory agencies have now licensed MSC-based drugs, although most of these approvals have been provisional or are based on underpowered studies (see 'Doubtful drugs').

In our view, the wildly varying reports have helped MSCs to acquire a near-magical, all-things-to-all-people quality in the media and in the public mind[3] — hype that has been easy to exploit. MSCs have become the go-to cell type for many unproven stem-cell interventions. The confusion must be cleared up.

What is needed is a coordinated global effort to improve understanding of the biology of the cells currently termed MSCs, and a commitment from researchers, journal editors and others to use more-precise labels. We must develop standardized analyses of gene expression, including on a cell-by-cell basis, and rigorous assays to establish the precise products of cell differentiation in various tissues. Such ▶

efforts could put an end to lingering questions about MSC identity and function, once and for all.

A CONTROVERSIAL CELL

The MSC concept dates to a 1991 paper[4] in which US biologist Arnold Caplan described the isolation of a type of stem cell found in bone marrow, building on reports by other groups (see, for example, ref. 5). Collectively, this work showed that certain cells in the supportive tissue of bone marrow (the stroma) could differentiate into cartilage, bone and fat, and provide some of the signals needed for haematopoietic stem cells (the immature precursors of all blood cells) to give rise to blood-cell types.

Over the next decade, there was an explosion in the number of tissue types in which MSCs were reported. And there was a similar jump in the diversity of cell types that MSCs were reportedly able to differentiate into. Yet by the early 2000s, it had become clear that separate labs were using different cell-surface markers to characterize MSCs. In 2006, a working group of the International Society for Cellular Therapy (ISCT) acknowledged the "inconsistencies and ambiguities" and recommended a new designation: multipotent mesenchymal stromal cells[6].

Despite the ISCT's recommendation, the stem-cell designation has proved strangely durable. Assays commonly used to evaluate a cell's 'stemness' are prone to misinterpretation. Many researchers have failed to take into account the developmental origins of the tissues they cite as sources of MSCs, or to observe the rigorous definition of a stem cell. Also, the very commonness of the MSC term seems to have consolidated its acceptance.

In the past few years, however, there has been further questioning of the MSC as a valid biological entity. A 2014 study[7] by researchers at the US Food and Drug Administration found almost no agreement in the molecular characterization of MSCs among the investigational new drug applications submitted to the agency. A 2016 study[2] likewise found that various cell populations from different tissues, all classed as MSCs, actually differ drastically in their gene expression and in their ability to differentiate. And last year, Caplan revealed that he no longer believes that MSCs are stem cells[1].

In that article, Caplan implores the scientific community to adopt yet another moniker: medicinal signalling cells. (According to Caplan, medicinal signalling cells, identified on the basis of cell-surface proteins and their ability to turn into other cell types *in vitro*, home in on sites of injury. There they secrete cocktails of proteins that modulate the immune response, reduce inflammation, promote wound healing and inhibit cell death.)

Since 1991, more than 32,000 Medline-indexed articles referring to "mesenchymal stem cells" have been published. Our reading of a subset of these — many hundreds over the past decade — suggests that the field continues to be a mess.

According to the literature, MSCs are most often isolated from bone marrow and adipose tissue (fat). They have also been identified in perinatal tissues — umbilical cord, Wharton's jelly (a gelatinous substance found in the umbilical cord), the amnion (the membrane surrounding the embryo) and the placenta — as well as in other sources, including baby teeth and menstrual blood. Some groups report that MSCs are most common in fat. Others state that they are associated with blood vessels throughout the body's connective tissues. Some note that MSCs are exceedingly rare; others say they are abundant.

Canonically, MSCs (including those from non-skeletal sources) are supposed to give rise to cartilage, bone and fat. But reports exist of their differentiation into muscle, endothelium and various cells of the heart, liver and kidney, as well as of the nervous and reproductive systems. Some researchers and clinical providers have even described MSCs as nearly pluripotent, meaning that they can differentiate into almost every cell type in the adult body. Moreover, there are countless claims of therapeutic uses for these cells in multiple unrelated diseases, ranging from arthritis and diabetes to Parkinson's disease and autism.

SALES PITCH

All of the disagreement in the scientific community about the nature of MSCs, and even about their existence, is almost certainly making it easier for businesses to sell treatments allegedly based on MSCs.

Direct-to-consumer marketing of unapproved stem-cell treatments for medical conditions has exploded in the past five years, particularly in the United States, Australia and Japan. A 2016 report[8] documented 351 US companies selling putative stem-cell treatments direct to consumers; almost half refer to MSCs in their marketing materials.

Businesses have been quick to capitalize on the conflicting claims in the literature[9]. To convey that MSCs can treat a wide range of diseases and injuries, firms selectively highlight those articles suggesting that MSCs are easy to harvest and can give rise to other cell types, or those indicating that the cells secrete all sorts of healing factors. (The large body of research indicating that tissue-specific stem cells have more limited roles is overlooked.)

Interestingly, some publicly traded companies have shown increasing reluctance to define their products as mesenchymal stem cells. Cytori Therapeutics in San Diego, California, a developer of devices for harvesting and processing cells from patients, has begun to describe its target cell population as 'adipose-derived regenerative cells' instead of calling them MSCs.

SOLUTIONS IN SCIENCE

What can be done to clear up the confusion? In our view, re-categorizing MSCs as 'stromal' or 'signalling' cells won't help.

Some researchers are following the ISCT recommendations, but judging by the thousands of papers on MSCs still being published every year, many are not. Meanwhile, Caplan's proposal could introduce more problems than it solves. There is strong evidence for the existence of a tissue-specific stem cell in bone-marrow stroma at least, albeit one with a limited ability to differentiate into other cell types. Also, the use of 'medicinal' instead of 'mesenchymal' could encourage the assumption that MSCs have broad therapeutic usefulness before robust evidence for this has been obtained.

We think that answers will be found in better science. After all, many researchers now doubt that a single multipotent stem cell (meaning one that can give rise to several other kinds of cell) is present throughout the adult body, thanks to the rigour and persistence of some stem-cell biologists.

The reliable identification of tissue-specific cells — stem cells or otherwise — should involve omics approaches, such as those designed to analyse the gene-expression patterns of a cell or its protein content, and rigorous assays to establish what a particular cell can differentiate into, either *in vivo*

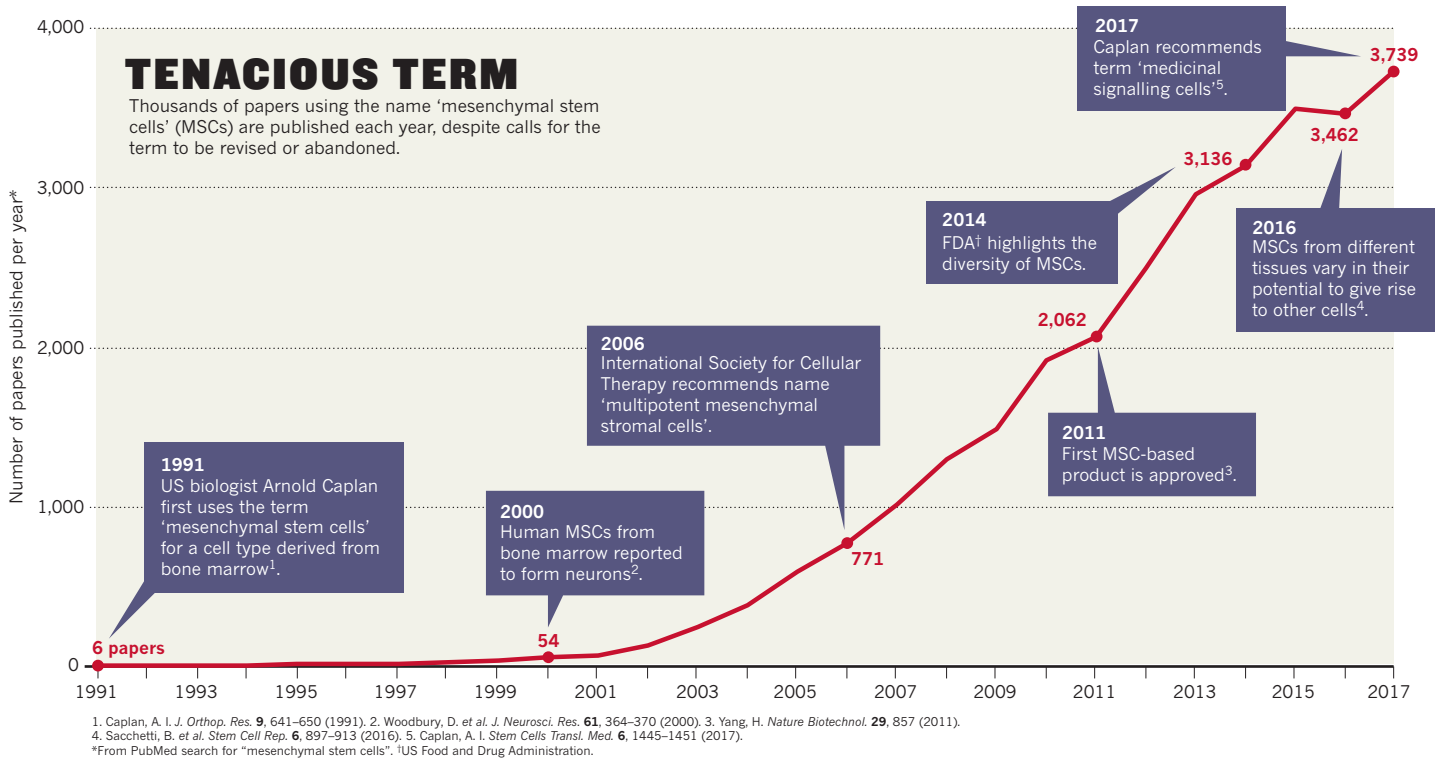
DOUBTFUL DRUGS

Clinical trials with MSCs fail to deliver

A move towards translational studies requires a robust understanding of the actual biological properties of the cell types currently called mesenchymal stem cells (MSCs).

So far, both the results and the quality of MSC-based clinical trials have been underwhelming. Take a 2014 meta-analysis of 49 trials using 'bone-marrow stem cells' (in many cases, 'bone-marrow MSCs') to treat cardiovascular disease, for instance. According to that analysis, the studies that scored better in terms of rigour were more likely to report less efficacy for MSC treatments than were those judged to be less rigorous[11].

Clinical studies using MSCs (or any stem cells) must adhere to the same standards of research design and oversight that apply to any responsible clinical trial before the cells are administered to human participants. **D.S., P.G.R. & L.T.**



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*From PubMed search for "mesenchymal stem cells". †US Food and Drug Administration.

or *in vitro*. (Historically, inconsistencies in surface markers and stains, or the use of unreliable morphological characteristics, have led to frequent errors when identifying cell types[10].)

Ongoing initiatives such as the Human Cell Atlas, an effort to characterize all cells in the body, could shed light on the cellular components of specific tissues. But, ultimately, resolving the MSC identity crisis is likely to require a focused undertaking by stem-cell biologists — similar to a series of projects conducted in the 2000s by the International Stem Cell Forum to characterize pluripotent stem cells. (The forum is a collaboration designed to support stem-cell research.)

As part of the push for better science, regulatory agencies and editors of influential stem-cell and general scientific journals will need to develop and enforce rigorous methodological standards.

DISPEL THE MSC MYTH

Meanwhile, the community needs to stop subsuming multiple cell types under one catch-all phrase.

Scientific societies, such as the International Society for Stem Cell Research (ISSCR) and national stem-cell organizations, should consider whether MSC studies ought to be presented at stem-cell conferences under the MSC umbrella. Clinical-trial registries, such as clinicaltrials.gov, should also exercise heightened scrutiny over studies in which MSCs are described as the investigational product. (Hundreds of such studies are already listed on international clinical-research databases.) Groups studying tissue-specific stem cells should be encouraged to rethink their choice of terms. Both bone marrow and fat, for example, contain stem-cell populations that help to maintain these tissues. And referring to these as skeletal or adipose stem cells could help to vanquish the myth of a near-ubiquitous, all-powerful adult MSC[2]. Certainly, studies of poorly characterized cellular miscellanies should not be accepted without rigorous evidence of multipotency and self-renewal.

"In most cases, rigorous preclinical studies of these cells are limited or non-existent."

Journal editors, editorial boards and reviewers should similarly be more exacting when accepting or approving MSC reports for publication in journals. And funding bodies must consider whether studies using MSCs qualify for funds earmarked for stem-cell research.

Journalists can also play an important part in combating MSC misconceptions. When reporters write stories about clinics selling 'mesenchymal stem-cell treatments' and other purported stem-cell therapies, they should inform their audiences that scientists debate whether such cells are, in fact, stem cells. They should also make it clear that, in most cases, rigorous preclinical studies of these cells are limited or non-existent.

Meanwhile, organizations such as the ISSCR, the ISCT and national stem-cell societies should ensure that guidebooks, educational videos and other communication tools designed for people receiving 'adult stem-cell therapies' are updated to reflect the demise of the MSC as a viable concept.

Whatever the ultimate identity and biological activity of the cells formerly known as MSCs, the scientific principles that guide their clinical development and use must be the same as those for other new therapies: precision, validation, characterization, objectivity and the abandonment of terms and conceptual models that mislead more than they illuminate. ■

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