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# Perivascular – derived mesenchymal stem cells

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Abstract

Cells have been identified in post-natal tissues that when isolated from multiple mesenchymal compartments, can be stimulated *in vitro* to give rise to cells that resemble mature mesenchymal phenotypes such as odontoblasts, osteoblasts, adipocytes and myoblasts. This has made these adult cells, collectively called mesenchymal stem cells (MSCs), strong candidates for fields such as tissue engineering and regenerative medicine. Based on evidence from *in vivo* genetic lineage tracing studies, pericytes have been identified as a source of MSC precursors *in vivo* in multiple organs, in response to injury or during homeostasis. Questions of intense debate and interest in the field of tissue engineering and regenerative studies, are 1. Are all pericytes, irrespective of tissue of isolation, equal in their differentiation potential? 2. What are the mechanisms that regulate the differentiation of MSCs? To gain a better understanding of the latter, recent work has utilised ChIP-seq to reconstruct histone landscapes. This indicated that for dental pulp pericytes the odontoblast specific gene *Dspp* was found in a transcriptionally permissive state, whilst in bone marrow pericytes the osteoblast specific gene *Runx2* was primed for expression. RNA-seq was also utilised to further characterise the two pericyte populations and results highlighted that dental pulp pericytes are already pre-committed to an odontoblast fate based on enrichment analysis done indicating over-representation of key odontogenic genes. Furthermore, a ChIP-seq experiment on the PRC1 component RING1B indicated that this complex is likely involved in inhibiting inappropriate differentiation as it localised to a number of loci of key transcription factors that are needed for the induction of adipogenesis, chondrogenesis

or myogenesis. In this review we highlight recent data elucidating molecular mechanisms that indicate pericytes can be tissue specific pre - committed MSC precursors *in vivo* and that this pre-commitment is a major driving force behind MSC differentiation.

Keywords: pericytes, stem cells, plasticity, regeneration, epigenetics, mesenchymal stem cell, perivascular stem cell, mesenchymal stromal cell, tissue repair

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*Introduction*

Mesenchymal stem cells (MSCs) is the generic name (rightly or wrongly), commonly used to describe cells isolated from connective tissues (stroma), that *in vitro* exhibit properties of stem cells. The prototypical MSC described almost three decades ago, resides within bone marrow (Caplan 1991; Friedenstein et al. 1974; Pittenger et al. 1999). Since then, cells with similar immunophenotype (*in vivo* and *in vitro*), molecular, and functional properties have been described in multiple organs, including teeth (Gronthos et al. 2002; Gronthos et al. 2000; Miura et al. 2003; Sonoyama et al. 2008), dermis (Toma et al. 2001), adipose (Zuk et al. 2002), and muscle (Dellavalle et al. 2011). Functional (*in vitro*) characteristics include: their adherence to tissue culture plastic (Pittenger et al. 1999; Zhu et al. 2010), morphological similarity, multipotency and tri-lineage differentiation into mesodermal lineages including chondrocytes, adipocytes and osteoblasts when stimulated with a cocktail of factors (Beltrami et al. 2007; Covas et al. 2008; Huang et al. 2009). While the field was intensely focused on studying and exploiting the *in vitro* characteristics of MSCs, much less attention has been paid to identifying their *in vivo* origin, although certain observations have provided some important clues. To paraphrase Murray et al, there has been great focus on multipotency and tissue engineering and repair, but the native origin and physiological roles of MSCs *in vivo* have been greatly overlooked. The

*in vivo* counterpart of culture expanded MSCs remained largely unknown for many tissues (Murray et al. 2014).

Connective tissues (stroma), exist throughout the body and this suggests that their precursors are locally available in multiple tissues where they can act as a source of MSCs for homeostatic maintenance and repair of these structures. This would explain why MSCs can be isolated from almost all tissues (Bianco et al. 2001; Crisan et al. 2008a; Farrington-Rock et al. 2004; Shi and Gronthos 2003). In the late 1800s, a contractile cell population residing around capillaries was described by Charles Benjamin Rouget (Rouget 1873). These *Rouget* cells were then renamed by Karl Zimmermann to what today we refer to as *pericytes* (Zimmermann 1923), to better describe their perivascular location in close association with blood vessels. When considering their cell surface marker profiles, pericytes express many MSC "markers" (*in vivo* and *in vitro*) CD90, CD73, CD105 and CD44. In addition, other markers such as CD146, $\alpha$ smooth muscle actin ($\alpha$SMA), neural glial antigen 2 (NG2) and platelet derived growth factor receptor $\beta$ (PDGFR-β) have been associated with pericytes in multiple human and mouse organs including pancreas, placenta, skeletal muscle and adipose tissue (Caplan 2008; Crisan et al. 2008b). Pericytes isolated using these markers can give rise to adipocyte-like, osteoblast-like and chondrocyte-like cells *in vitro* using tailored tissue culture conditions (Caplan 2008; Crisan et al. 2012; Crisan et al. 2008a; Doherty et al. 1998). This has resulted in more similarities being identified between MSCs and pericytes (reviewed extensively in : Caplan et al. 2008, and Feng et al. 2010 ) leading many researchers to an acceptance that in most tissues, pericytes are precursors of MSCs (Covas et al. 2008; Schwab and Gargett 2007). A further source of evidence for this came from the observation that typical MSC cultures were capable of being established using blood vessels alone (Caplan 2007). Genetic lineage tracing studies performed using a number of Cre-recombinase mouse lines is providing *in vivo* evidence, showing that pericytes can give rise to mature MSC progenitors such as odontoblasts (Feng et al. 2011), osteoblasts (Supakul et al. 2019), and myoblasts (Dellavalle et al. 2007) (Figure 1). A question that remains unanswered however, is how a widely distributed differentiated cell type, a pericyte, can give rise to MSCs that will differentiate into tissue-specific cell

types depending on the tissue where they are located. Is this a result of niche originating signals or mechanisms more intrinsic that regulate appropriate choice of cell fate (Feng et al. 2010)?

*Evidence of MSC pre-programming*

Although MSCs from many tissues can differentiate into osteoblast-like cells, chondrocyte-like cells and adipocyte-like cells *in vitro*, they do not do this *in vivo* during homeostasis. Although a multipotent MSC may exist, no single cell has been definitively described that forms these three differentiated cell types *in vivo*. Several cell populations have been described that can undergo a form of multi-lineage differentiation *ex vivo*, but single cell lineage tracing *in vivo* is needed to definitively prove the existence of a multipotent stem cell (Chan et al. 2018; Mizuhashi et al. 2018).

Most MSCs when expanded *in vitro* show a certain homogeneity in their morphology, cell surface antigen profile, and differentiation potential when stimulated (Beltrami et al. 2007; Covas et al. 2008; Huang et al. 2009) . It has however, always been recognised that MSCs derived from a wide range of tissues are inherently different as suggested by *in vitro* experiments. Depending on where the cells are sourced from, they vary in their colony forming efficiency (Peng et al. 2008), their ability to influence the immune system (Hegyi et al. 2010), and most importantly their potential to differentiate down certain mesodermal lineages. MSCs sourced from umbilical cord blood are unable to differentiate into adipocytes (Peng et al. 2008) or myoblasts (Sacchetti et al. 2016). Conflicting reports exist with regards adipose derived MSCs and their ability to differentiate down osteogenic lineages (Azzaz et al. 2014; Covas et al. 2008; James et al. 2012a; James et al. 2012b; James et al. 2012c). It is likely that the outcomes of these experiments are context dependent on the adipose tissue depot used. What is clear from our observations and that from others such as Sacchetti et al (2016), is that pericytes are most likely to yield cells appropriate to the tissue compartment from where they were isolated. It is important to note that the vast majority of experiments performed on MSCs describing their multipotency, have been carried out with cells propagated *in vitro*. This could impact the

behaviour and innate plasticity of these cells. Therefore, more focus should be placed on studies where a strong emphasis is placed on observing these cells as close to their *in vivo* context as possible.

What we have observed is that mechanisms for faithful propagation of the appropriate lineage identity are established in these cells *in vivo* (Figure 2). To investigate these mechanisms, we isolated pericytes from mouse incisor and long bone. The selection of these cells was based on their proven ability to differentiate into two different mineralised cell types (odontoblasts and osteoblasts respectively) and also their different embryonic derivation, cranial neural crest and pulp mesoderm respectively (Arthur et al. 2008; Dennis and Charbord 2002).

Isolated pericytes were profiled using a number of next generation sequencing (NGS) approaches, including RNA sequencing (RNA-Seq) and chromatin immunoprecipitation followed by sequencing (ChIP-Seq), to reveal extensive differences in the transcriptomes and histone landscapes of these cells (Yianni and Sharpe 2018). What emerged was that fresh pericytes from tooth pulp and long bone marrow shared expression of genes relating to homeostatic processes and those involved in physical (adhesion) and molecular (signalling) interactions with the underlying vasculature. Epigenomic profiling revealed that the majority of genes in transcriptionally active chromatin regions, identified by the abundance for trimethylation of lysine 4 on histone 3 (H3K4me3) (Dennis et al. 2003; Karlić et al. 2010; Zhang et al. 2015) were shared between both pericyte populations and were statistically enriched for genes involved in regulation and maintenance of cell adhesion. The majority of the genes found in these H3K4me3 regions were confirmed to be actively expressed by RNA-seq profiling, this being a reflection of these pericytes attachment to blood vessels. Focusing on gene expression differences between the two populations, a gene set enrichment (GSEA) analysis (Subramanian et al. 2005)unexpectedly revealed that dental pulp residing pericytes were enriched for a number of genes involved in odontogenesis. The most striking feature in these incisor pericytes is a low baseline expression of *Dspp*, a gene expressed in odontoblasts, a highly specialised cell type that can differentiate from pericytes. This was also evident when interrogating the *Dspp* locus for the

prevalence of a transcriptionally permissive or repressive histone environment. Incisor pericytes possessed a sharp peak of H3K4me3 at the transcription start site (TSS) of *Dspp*, while their bone marrow counterparts did not. Mouse incisors grow rapidly, (1.0 mm in 2 days) (An et al. 2018) and the transcriptionally open state of chromatin at the promoter of Dspp is indicative of a stem cell primed to differentiate. In the BM pericyte RNA-seq datasets we did not detect significant expression of genes relating to osteoblast specification. However when investigating the epigenetic landscapes of these BM pericytes we found that the promoter of the *Runx2* transcription factor, which is the master regulator and first step towards osteoblast differentiation (Gaur et al. 2005; James 2013) was in a bivalent state. This locus was abundant in both active and repressive histone post translational modifications as shown by ChIP-seq (Yianni and Sharpe 2018) (Figure 3). This observation is intriguing, since it is known that bivalent promoters mark genes that are poised for activation should the cognate upstream regulator (e.g a transcription factor) or stimulating signal become available (Bernstein et al. 2006; Dennis et al. 2003; Gan et al. 2007). This prompted further investigation into the epigenetic status of master regulators governing commitment for myogenesis, chondrogenesis and adipogenesis. To do this we performed a ChIP-seq experiment to identify the genomic binding sites for the catalytic subunit of polycomb repressor complex 1 (PRC1), RING1B. RING1B is an E3-ubiquitin ligase which allows PRC1 to polyubiquitinate lysine 119 of histones H2A (H2Aub119) (Stock et al. 2007). This causes further compaction of that genomic locus and predominantly leads to stable repression of genes within that locus due to a number of reasons, including physical exclusion of transcription factors from subsequent binding (Ren et al., 2008; van Arensbergen et al., 2013; Vidal, 2009). (Ren et al. 2008) (van Arensbergen et al. 2013) (Vidal 2009)What these datasets revealed was that a number of genes necessary to drive pericytes down inappropriate (for their organ) lineages were bound by RING1B and repressed. These included *Myf5*, *Pax3* and *Myod* for myogenesis, *Osr1*, *Runx3*, *Osr2*, *Nfib*, *Scx* and *Sox9* for chondrogenesis, and *Cebpa* and *Cebpb* for adipogenesis. This highlighted that *in vivo*, bone marrow and incisor pericytes are lineage restricted by previously undescribed mechanisms to give rise to pre-programmed MSCs that can then differ

tiate into organ-specific cell types (Figure 3). An important question this raises is are these epigenetic mechanisms a bi-product of niche originating regulatory events, potentially driven by signalling cascades, or are these mechanisms innate in the pericytes themselves? To further investigate this, FACS isolated pericytes from both incisor pulp and bone marrow were expanded *in vitro* using basal medium lacking any stimulating factors, thereby removing any potential niche - originating signals and tailored differentiation cocktails. Total RNA was collected from these cells after 31 days and pericytes isolated from incisor pulp were found to have upregulated expression of *Dspp*, whilst pericytes isolated from bone marrow had upregulated expression of *Runx2*, as compared to their fresh *in vivo* counterparts (Figure 4). These *in vitro* expanded pericytes did not upregulate marker genes of any other mesodermal lineage. This observation indicates that although *in vitro* these pericytes can be driven to produce muscle, mineral or fat, this is a by-product of their exogenous stimulation and not their endogenous lineage plasticity. These cells carry an epigenetic and transcriptomic program that persists even in culture that restricts them to a particular lineage, in this case enforcing an odontoblast or an osteoblast differentiation program respectively (Figure 4). This should not be surprising since *ex vivo* differentiation of bone marrow derived and tooth pulp derived MSCs clearly indicates tissue of origin -specific differentiation (Gronthos et al. 2000). This data also accurately mirrors the *in vivo* observations from lineage tracing studies showing the contribution of dental pericytes to odontoblasts (Feng et al. 2011; Vidovic et al. 2017)and bone marrow pericytes to osteoblasts (Supakul et al. 2019) and not to other lineages within the same organ.

A recent study has led to some confusion regarding the role of pericytes as MSC precursors where lineage tracing with *Tbx18*, a gene reported to be expressed by pericytes, showed no evidence of a differentiated cell contribution in certain tissues including fat, brain and heart (Guimarães-Camboa et al. 2017). In addition to already published comments on this study, our transcriptome datasets show no expression of *Tbx18* in cells identified as pericytes (Yianni and Sharpe 2018).

It is important to note that researchers outside the mineral biology field have also provided evidence indicating that *in vivo,* pericytes are not plastic, but tissue specific pre-committed precursors of MSCs, although the molecular basis of this restriction was not investigated. In a recent study, Sacchetti and colleagues (Sacchetti et al. 2016) established MSC cultures from pericytes sourced from tissues including bone marrow (BM), skeletal muscle (MU) and perinatal cord blood (CB). These perivascular cells were FACS isolated from fresh human tissues with the criteria of being negative for pan-endothelial and pan-haematopoietic marker but positive for MCAM ( $CD34^-$ / $CD45^-$ / $CD146^+$ ). These cells gave rise to MSC colonies *in vitro* under basal culture conditions that do not stimulate differentiation. Using gene-expression profiling the authors determined the transcriptional signature of these cells and performed unsupervised hierarchical clustering and principle component analysis. An ANOVA-based analysis determined that these cells are radically divergent from each other in terms of the genes they express when comparing the same "MSCs" isolated from different anatomical locations. They showed that the MU cells express the lineage specific myogenic transcription factor PAX7. Interestingly, BM MSCs express a number of genes associated with haematopoietic support something that was also validated in our recent epigenetic studies. Sacchetti et al, noted that the pericytes they prospectively isolated ( $CD34^-$ / $CD45^-$ / $CD146^+$ ) when transplanted heterotopically using an osteoconductive carrier, formed bone and also established a haematopoietic microenvironment while MU and CB MSCs did not. They further showed that MCAM/CD146 expressing stromal cells from different human tissues differed radically from their BM counterparts in differentiation potency and transcriptional profile, reflective of their different developmental origins. While BM derived MSCs/pericytes are natively skeletogenic but not myogenic, muscle derived MSCs/Pericytes are inherently myogenic but not natively skeletogenic and represent a subset of cells with functional features of satellite cells (Figure. 5) (Sacchetti et al. 2016). Multiple studies independently corroborate that MSCs and their precursors, residing in a wide range of tissues, are very similar, but they are clearly not identical populations. It was first thought that the tissue specific microenvironment could be a likely source of this variation, but evidence highlighted here would argue

that this is something intrinsically encoded within the molecular memory of these cells. When considering the differentiation capabilities of these cells (based on *in vitro* studies) it makes biological sense that molecular checkpoints would be in place to prevent non-specific differentiation *in vivo*. It could be argued that with age these molecular mechanisms could be perturbed as is suggested in tissues of aging patients or experimental animals where pericytes increasingly contribute to scarring and fibrosis as opposed to regeneration and repair (Goritz et al. 2011; Greenhalgh et al. 2013; Sundberg et al. 1996).

*Considerations for the future*

Recent publications in the field of MSC biology highlight a number of important concepts that have been largely overlooked or understudied. There is a widely shared acceptance that pericytes and MSCs are ubiquitous in the mesenchymal compartment of tissues, and regardless of the tissue they are sourced from, can be expanded to give a skeletogenic, adipogenic or myogenic phenotypes depending on how they are stimulated. Data discussed here clearly demonstrate that these ubiquitous cells differ in their *in vivo* transcriptomes/epigenomes and are not uniform in terms of their lineage commitment: they represent subsets of tissue specific lineage committed precursors.

A number of important points need to be raised; at this stage it is unclear how differences in cell surface marker expression might possible mirror functional heterogeneity *in vivo*. When taking into account the total pericyte content of a tissue, one subset of cells might be pre-destined to differentiate into mature mesodermal cells while others are confined to functional properties relating to the maintenance of the underlying vasculature. Single cell RNA-seq studies are being utilised to better understand the population architecture of pericytes within a given organ (He et al. 2018; Tikhonova et al. 2019; Zeisel et al. 2018). In the brain for example, single cell transcriptomics has identified three different populations of pericytes (Zeisel et al. 2018)

These studies can be used as a way to design lineage tracing strategies to investigate the contribution of these sub-types to other cell types *in vivo*. Data discussed here have implications for a number of

fields including stem cell biology, regeneration, and also tissue engineering. In approaches that require the use of MSCs in a scaffold to facilitate repair *in vivo*, not all sources of primary cells will be equivalent in their ability to regenerate the tissue of interest.

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Figures and Legends:

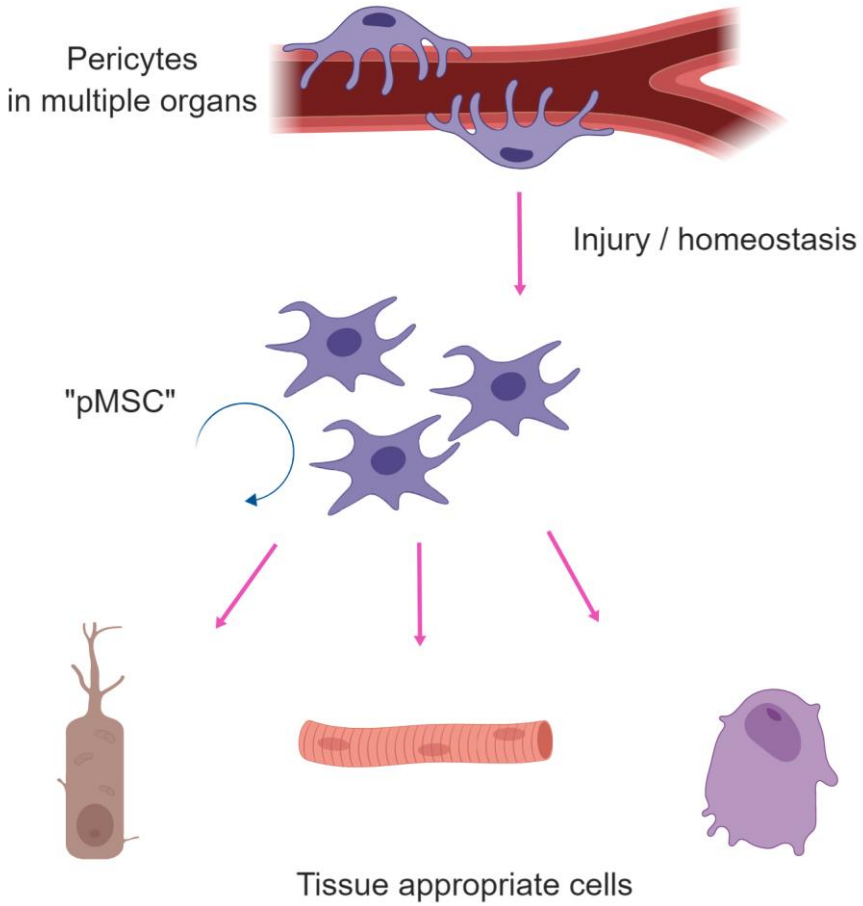


**Figure 1**. ***Pericyte injury/repair response in vivo***. In response to injury, or as part of maintaining tissue homeostasis, pericytes detach from the blood vessel wall and act as *bona fide* mesenchymal stem cells we will refer to as pMSCs, to distinguish them from MSCs that are not pericyte derived. pMSCs can proliferate and move to the area of injury where they differentiate into tissue specific cells to facilitate repair.

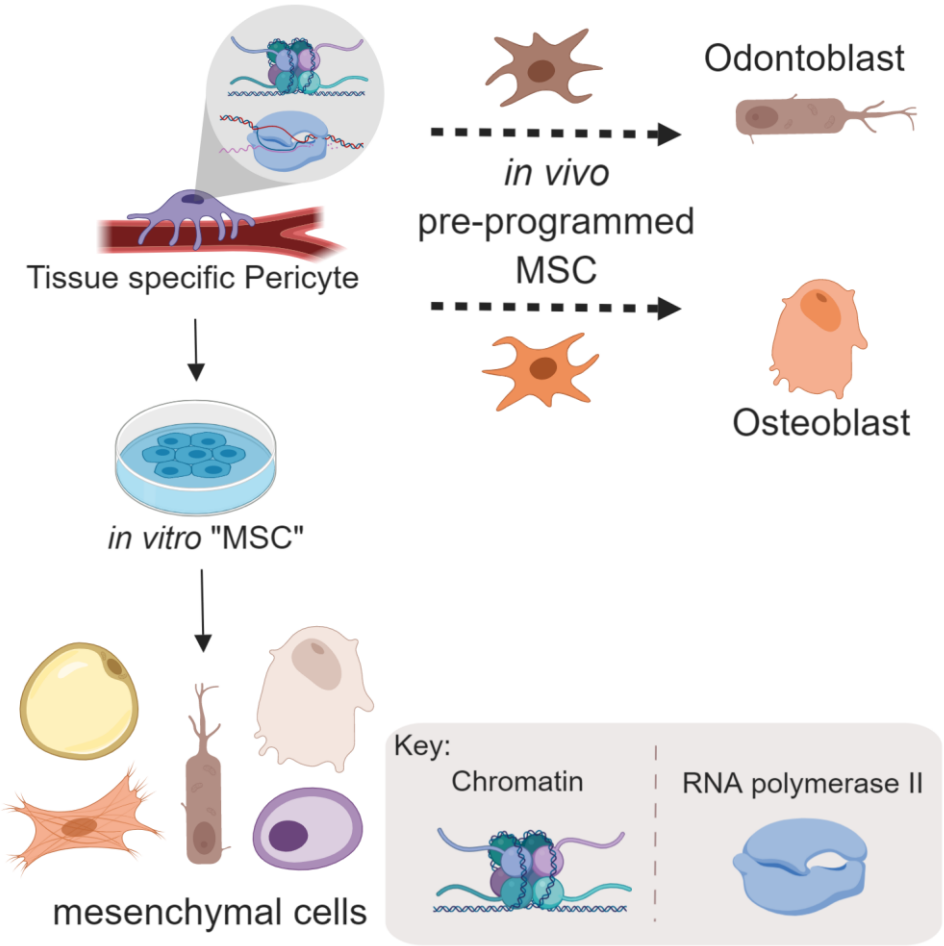


**Figure 2**. ***Pericyte differentiation potential, in vitro and in vivo***. *In vivo*, pericytes have a tissue - specific epigenome and a transcriptome that is restricting their differentiation. Upon receiving a signal, these pericytes detach and adopt an MSC phenotype that is pre-programmed to give rise to cells that will facilitate repair. *In vitro*, isolated pericytes will adhere to tissue culture plastic and give rise to characteristic looking MSC cultures. These can be directed to differentiate into any mesenchymal lineage using tailored stimulating factors that partially alter the inherent epigenetic/transcriptomic programs of these cells. As previously shown, if left unstimulated, these cells will upregulate lineage specific genes of specialised cells appropriate to their tissue of residence(Yianni & Sharpe, 2018).

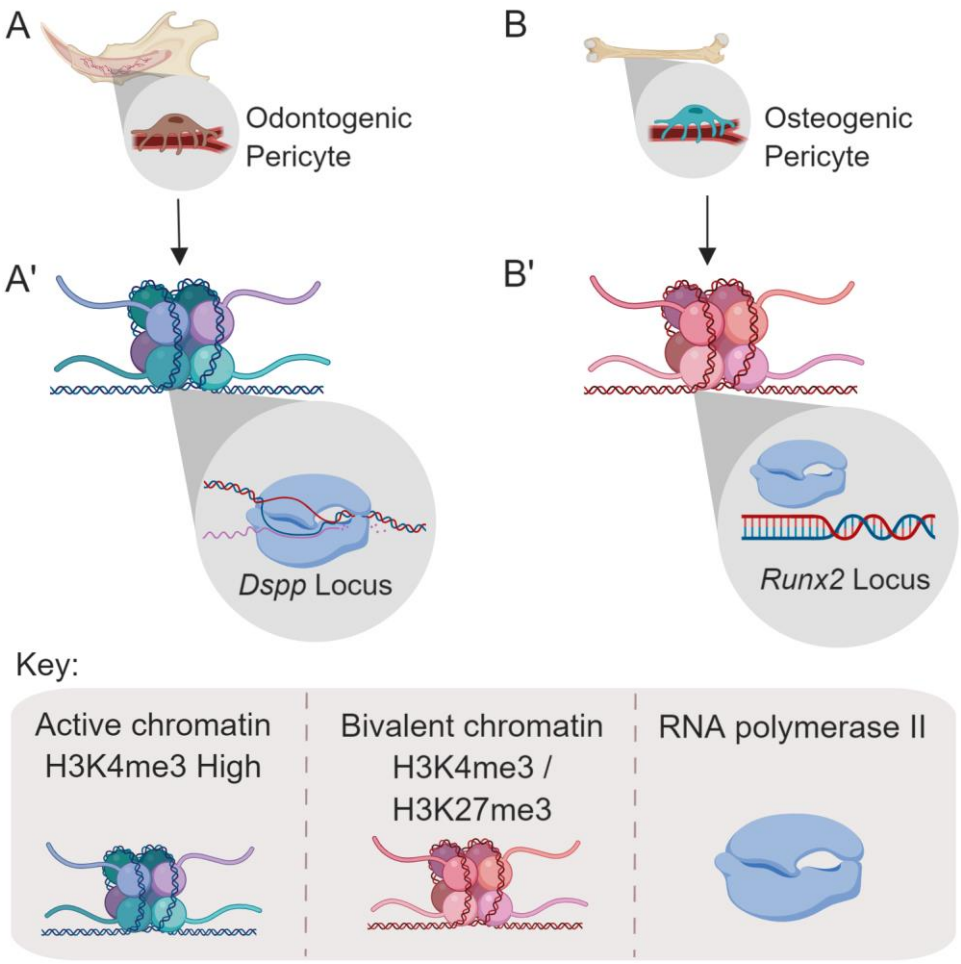


**Figure 3.** ***Lineage priming in vivo*.** A molecular method has been identified in dental and bone marrow pericytes that contributes to organ specific lineage commitment before pericytes detach from the basal membrane of blood vessels. In incisor pericytes a transcriptomically permissive chromatin structure is in place (high in H3K4me3) that allows transcription of *Dspp* to take place via the recruitment of RNA polymerase II (A). In contrast, while *Runx2* is not expressed in bone marrow pericytes, we have identified that it is positioned in a bivalent chromatin environment that makes it amenable to transcription once the upstream cognate transcription factors become available (B).

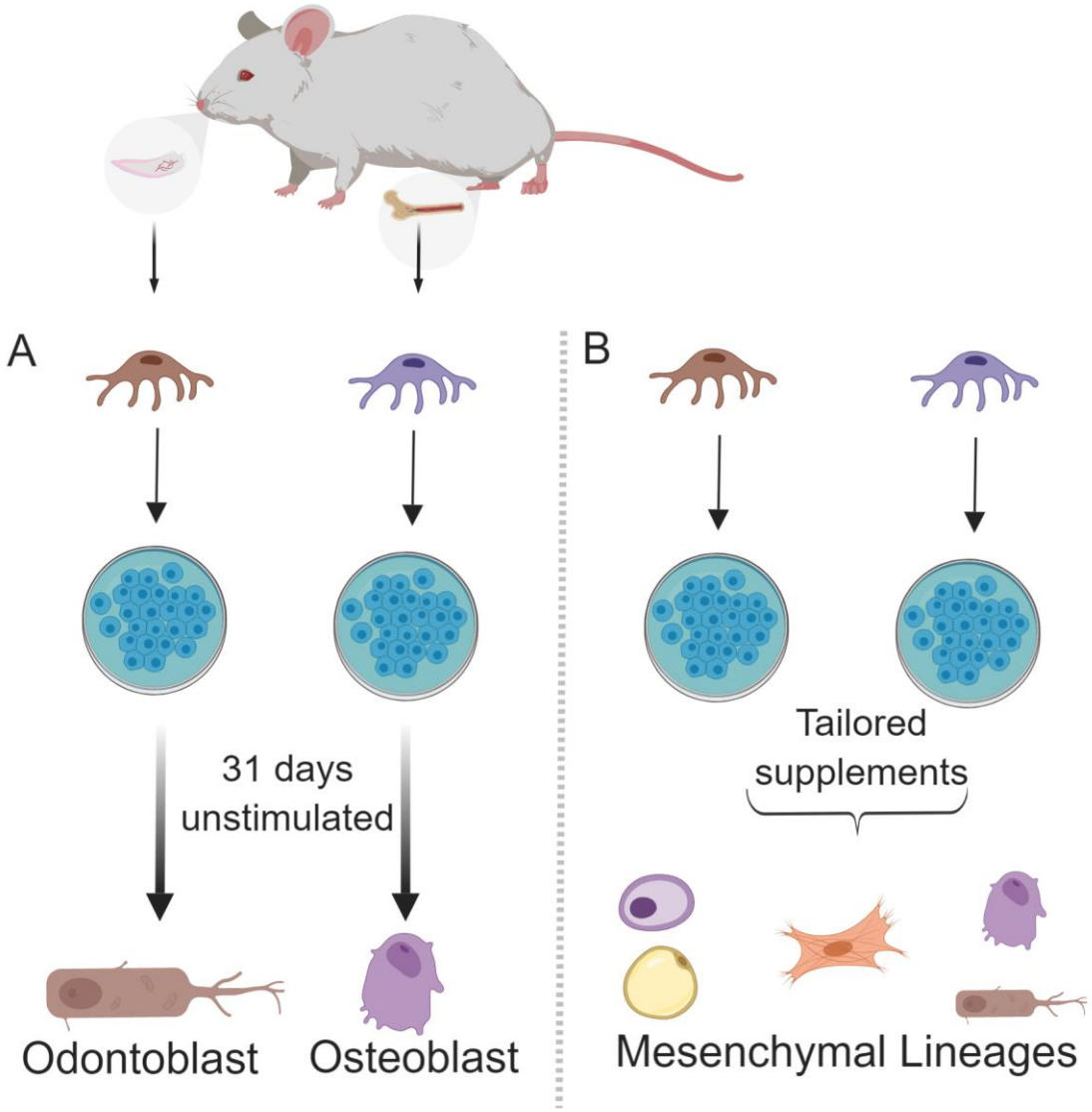


**Figure 4**. ***Lineage Restriction persists in vitro.*** Fresh pericytes isolated from incisor and long bone will readily adhere to tissue culture plastic and give rise to conventional MSC cultures. If they are left to proliferate and mature in the absence of stimulating factors, they will upregulate genes for the lineage specific to their tissue of origin (A). In contrast, most experiments perturb (or ignore) this innate MSC differentiation bias. These cells are driven down non-specific cell lineages by the addition of tailored supplements to give rise to mature, terminally differentiated, mesenchymal derivatives.

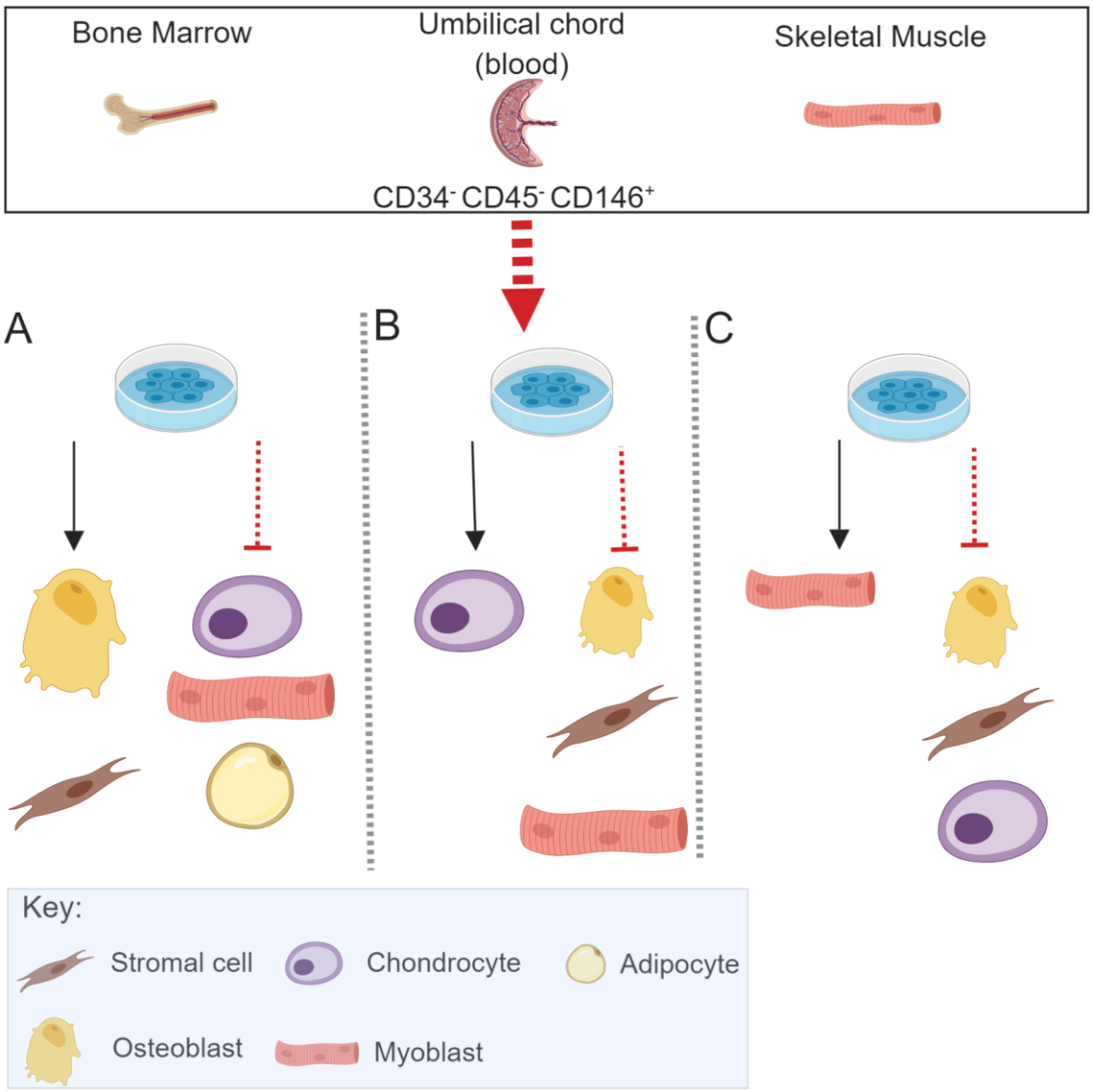


**Figure 5.** ***Pericyte MSCs as pre-programmed precursors.*** Sacchetti et al. isolated pericytes ( $CD34^-$ / $CD45^-$ / $CD146^+$ ) from human bone marrow (BM), post-natal umbilical cord blood (CB), and skeletal muscle (MU) which gave rise to traditional MSC cultures *in vitro*. These MSC cultures could only differentiate into bone producing osteoblast or haematopoietic supporting stromal cells if they were derived from BM (A). CB MSCs could only be differentiated into chondrocytes (B) and MU MSCs only into myoblasts (C). Adapted from (Sacchetti et al. 2016)