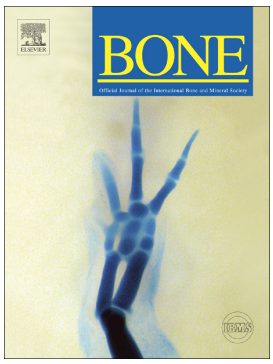

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Epigenetic mechanisms driving lineage commitment in mesenchymal stem cells

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Abstract

The increasing application of approaches that allow tracing of individual cells over time, together with transcriptomics and epigenomics analyses is changing the way resident stromal stem cells (mesenchymal stem cells) are viewed. Rather than being a defined, homogeneous cell population as described following *in vitro* expansion, *in vivo*, these cells are highly programmed according to their resident tissue location. This programming is evidenced by different epigenetic landscapes and gene transcription signatures in cells before any *in vitro* expansion. This has potentially profound implications for the heterotypic use of these cells in therapeutic tissue engineering applications.

Introduction

The prototypical mesenchymal stem cell (MSC), isolated from bone marrow (BM) was described over 3 decades ago by Friedenstein [1]. This characterisation was later expanded to include a cell type, isolated from connective tissue stroma that can exhibit stem cell properties *in vitro* [2, 3]. These observations led many researchers to identify cells in multiple organs that had a similar immunophenotype and characteristics *in vitro* of MSCs in that they could be differentiated to form osteoblast-like, chondrocyte-like, and adipocyte-like cells. Tissues included, connective tissue of teeth [4, 5], muscle [6], adipose [7], dermis [8], heart and liver [9]. These cell populations expressed a number of the by now established MSC gene markers and all shared some common characteristics including morphology, adherence to tissue culture plastic, and trilineage differentiation [9, 10]. The ease of

isolation, expansion, and the ability to differentiate these cells into various mesodermal derivatives placed 'MSCs' on a pedestal in the fields of tissue engineering and regeneration. What was largely overlooked at the time was the nature of the *in vivo* counterparts of these culture expanded MSCs. Today we know that connective tissues (stroma) exist throughout the body suggesting that when MSCs are needed (*in vivo*) for repair/regeneration, their precursors are locally available to provide these cells. This hypothesis can explain how MSCs (identified retrospectively *in vitro*) can be isolated from the stromal of practically all tissues [11-13].

A decade after Friedenstein, Charles Benjamin Rouget identified a population of contractile cells residing around capillaries [14]. Karl Zimmermann, prompted by the close association of these cells with the vasculature renamed them to *pericytes* [15]. Pericytes intrigued researchers since they appeared in practically all vascular organs, would adhere to tissue culture plastic, had a cell surface marker profile both *in vivo* and *in vitro* similar to that of "MSCs" and could also undergo trilineage differentiation under guidance of appropriate tissue culture supplementation [16, 17]. Pericytes can be isolated from both murine and human tissues using multiple markers (shared with *in vitro* MSCs) such as CD90, CD73, CD105, and CD146 [10, 18]. Further validation indicating pericytes are the *in vivo* precursor of cultured MSCs arose from the fact that traditional "MSC" cultures could be set up by culturing blood vessels alone [19]. Such observations led to the acceptance by the community that pericytes are the precursors of MSCs in many tissues. In order to add more biological evidence reinforcing the link between pericytes and MSC precursors, genetic lineage tracing has been employed to follow the fate of pericytes during development, repair, and regeneration *in vivo*. Such studies performed using a combination of Cre-recombinase mouse lines provided evidence that pericytes *in vivo*, give rise to mature MSC progenitor cell types such as myoblasts [20] osteoblasts [21] and odontoblasts [22]. These observations raise two questions of intense interest: Are all pericytes, irrespective of their anatomical origin equal in their differentiation potential? What mechanisms are employed to prevent inappropriate differentiation of pericytes *in vivo*? To date, no single cell has been identified that will definitively give rise to osteoblasts, chondrocytes and adipocytes *in vivo* that would support the concept that a multipotent mesenchymal stem cell exists in vivo [23, 24]. To date most studies have focused on how to functionally convert (in vitro) MSCs to

differentiated progenitors that could produce proteoglycans, or collagen type II producing cells. Utilising signalling pathways such as canonical hedgehog signalling or BMP signalling that are recognised potent drivers of mesenchymal fates [26-28] traditional culture induction techniques have been employed to convert MSCs into desired cell types. Illustrative examples of this include addition of exogenous growth factors such as BMP-2 to induce osteogenesis or BMP-7 to induce chondrogenesis in vitro [29]. Some authors even explored gene transfer into MSCs of potent chondrogenic regulators such as Indian hedgehog as a potential method of inducing a desired cell fate such as chondrogenesis [30]. These methods to some extent result in the desired outcome in vitro. What is unknown is how they influence the epigenome of these cells when bringing about a change in transcriptional output reflective of the desired differentiated cell state.

Mechanisms regulating MSC differentiation

Broadly speaking, differentiation of cells (MSCs or otherwise) relies on the activation of sets of genes responsible for a mature phenotype (morphological, functional etc), and repression of genes that confer stemness [25]. Changes in global transcriptional output are driven by epigenetic factors regulating how the genome interacts with the transcription machinery of the cell [26]. These epigenetic factors themselves are tuneable and accordingly triggered by incoming molecular signals or intrinsic cellular memory both of which are utilised to direct cell fate and appropriate differentiation [26]. “Epigenetic changes” refer to reversible changes (often heritable, through mitosis) that result in modulating gene expression without modifying the underlying nucleotide sequence [27]. Epigenetic changes broadly fall under the umbrella of 3D chromatin confirmation changes, DNA methylation, and post-translational modification of histones [28-31]. These mechanisms play a pivotal role in enforcing appropriate transcriptional networks during embryonic development but also during physiological tissue homeostasis. Alteration of the chromatin landscape regulates gene expression by facilitating opening of DNA (termed euchromatin) or by condensing DNA (heterochromatin). Euchromatin allows transcription to take place as the underlying DNA sequence is accessible to transcription factors and the necessary transcriptional machinery. Heterochromatin represses transcription partly by compaction of DNA, thereby abrogating transcription factors from being able to

access the relevant promoters. These and various additional mechanisms of actions have been described extensively elsewhere [25]. What is important to highlight for the purpose of this review is that trimethylation of lysine 4 of histone H3 (H3K4me3) marks euchromatin and gene activation. In contrast, H3K27me3 signals heterochromatin and gene repression [32, 33]. Once these marks are in place they can direct gene expression by modulating accessibility to gene promoters and therefore obstructing (or facilitating) recruitment of other transcriptional regulators or additional chromatin modifying enzymes [34-36].

Seeing how relatively understudied these mechanisms are in MSCs (or their precursors) *in vivo* we sought to understand how such epigenetic mechanisms could influence cell identity and bias subsequent differentiation potential. Various reports have been made regarding MSCs behaving differently *in vitro* based on tissue of isolation, therefore to avoid investigating potential artefacts of *in vitro* expansion we focused on characterising the histone landscapes of freshly isolated perivascular cells from bone and tooth [37].

Pericytes as pre-programmed MSC precursors *in vivo*

In vivo, pericytes play a key role in maintaining vascular integrity and ensuring function of the underlying endothelium. They do this by providing mechanical and trophic support via the secretion of angiogenic factors [38-40]. In addition to their indispensable support of the vasculature, pericytes actively contribute to tissue homeostasis and repair in a number of tissues [41-45].

In vivo, pericytes/MSC precursors detach from blood vessels, proliferate and move towards the site of tissue damage. These cells can then differentiate into mature mesodermal cell types that are capable of facilitating repair (eg. osteoblasts, odontoblasts etc) (Fig. 1A-C). This tightly regulated differentiation into a specific cell type appropriate to facilitate a repair makes physiological sense, since inappropriate differentiation could have severe implications. Indeed, fibrosis could be considered inappropriate MSC differentiation into fibroblasts [46-49]. Perhaps counterintuitively, this is what is stimulated to occur *in vitro* when MSCs are stimulated to differentiate (Fig. 1D).

To investigate potential differences that could contribute to control of *in vivo* differentiation, pericytes from mouse incisor dental pulp and bone marrow were compared. These organs were chosen since they are densely vascularised and resident pericytes give rise to mineral forming cells (odontoblasts or osteoblasts) in response to injury or during organ homeostasis [21, 22]. In addition, the two organs are derived from distinctive progenitor populations of the developing embryo, cephalic neural crest and mesoderm respectively [50-52]. The XLacZ4 transgenic mouse line in which pericytes constitutively express the *lacz* transgene was used [53]. To enable efficient isolation of pericytes by florescence activated cell sorting (FACS) (Fig. 2A). The cells were then collected and used to construct histone landscapes using chromatin immunoprecipitation followed by sequencing (ChIP-seq), and transcriptomically profiled using bulk RNA-sequencing (Fig. 2B). ChIP-seq was performed using antibodies targeted against trimethylated lysine 4 on histone 3 (H3K4me3) and trimethylated lysine 27 on histone 3 (H3K27me3). These were chosen to identify genomic regions enriched for euchromatin, heterochromatin and bivalent chromatin [54-56]. The primary observation was that both transcriptomically and epigenetically, these two populations are largely identical as the analysis returned similar enrichment patterns in both. This was not surprising since they were isolated using the same specific marker from two different mineral forming organs of the same mice, with their only difference being their anatomical separation. In line with their pericyte function, cells from both organs expressed an overwhelming number of genes relating to blood vessel maintenance and function. This was mirrored in both the RNA-seq and ChIP-seq datasets. Upon deeper analysis of the data, incisor pericytes were observed to be inherently odontogenic and even prior to leaving the blood vessel they expressed a number of odontogenic genes that would only be expected in odontoblasts, something which was not evident in their bone marrow counterparts for osteogenic genes. *Dspp* encoding two odontoblast specific proteins, dentin sialoprotein and dentin phosphoprotein was expressed in incisor pericytes and the promoter of *Dspp* was confirmed to have an enrichment peak for H3K4me3 while bone marrow pericytes lacked a euchromatin region at this locus and expressed no odontogenic signatures (SFig. 1) [37, 57]. Contrastingly, bone marrow pericytes revealed a gene expression fingerprint that mirrored their association with the haematopoietic stem cell niche as demonstrated by their expression of various cytokines and chemokines. Seeing that incisor pericytes were

biased toward an odontogenic cell fate we wanted to explore if bone marrow pericytes had a similar inclination towards an osteoblastic cell fate. Investigating the histone landscapes present at the *Runx2* locus, a key osteoblast fate specifying transcription factor [58, 59] revealed that it was in a bivalent chromatin state. A state characterised by pronounced enrichment of both active (H3K4me3) and repressive (H3K27me3) histone marks that identifies genes primed for future activation upon cell stimulation by appropriate signals [60-62]. This bivalent state was not evident at the loci of other lineage specifying transcription factors. Supplementary to this, gene products used traditionally to identify adipogenic and chondrogenic cell fate (*Pparg* and *Col2a1* respectively) were enriched for heterochromatin, while the late osteogenic gene *Ibsp* (encoding bone sialoprotein) was devoid of this mark indicating that it was amenable to transcription once the cognate transcription factor(s) become available. Analysis of the genetic loci encoding a number of mesodermal lineage specifying transcription factors showed that they were stably repressed as they were targets of the polycomb-repressor complex 1 (PRC1). The PRC1 complex being widely recognised as a major epigenetic modulator that enforces stable repression of transcription by compacting chromatin, thereby preventing access of RNA polymerases and transcription co-factors from accessing transcription start sites of genes [63-65]. ChIP-seq for the E3-Ubiquitin ligase RING1B showed that it actively targeted loci of genes specifying a number of cell fates. This was the case for *Cebpa* & *Cebpg* needed to specify an adipogenic cell fate [66], *Sox9*, *Osr1*, and *Osr2* [67, 68] required for a chondrogenic cell fate, and *Myf5*, *Pax3* and *Myod1* [69, 70] required for induction of a myogenic cell fate. This enrichment of RING1B was not seen at the loci of *Runx2* and *Ibsp,* thereby signifying that these were amenable for transcription. (Fig. 3A). To further test the above, fresh BM pericytes were FACS isolated and cultured for 31 days in basal medium lacking any growth factors or stimulants. These cells upregulated expression of *Runx2* but did not express other lineage markers that were found to be in heterochromatin dense regions of the genome (Fig. 3B) [37]. The study showed that *in vivo*, pericytes/MSCs are already pre-committed down a mesenchymal lineage appropriate to their anatomical location. In addition, if left unstimulated *in vitro*, these pMSCs will tend to adopt gene expression characteristics of their anatomical origins.

This does not appear to be a phenomenon only applicable to mouse MSCs. In a study performed on human specimens, Sacchetti et al (2016) isolated perivascular

cells ($CD146^+/CD34^-/CD45^-$) from umbilical cord blood, muscle, periosteum and bone marrow [71] then subjected cultures of these cells to RNA-seq. The subsequent gene expression profiling revealed that the cells have distinct transcriptomic signatures that are accompanied by very discreet differentiation capabilities. Muscle perivascular CD146+ cells have a transcriptome that is over-represented for tissue-specific genes. These include genes that regulate muscle contraction, and also muscle development. These could not be detected in CD146+ cells isolated from other organs utilised in this study. They also demonstrate that these cells will readily form myofibres which express classical mature muscle cell markers, in a matrigel assay. To assay the differentiation capacity of these cells *in vivo*, CD146+ cells were injected into the tibialis anterior of immunocompromised mice that had been previously been injected with cardiotoxin (24 hours prior) to induce acute skeletal muscle damage. Human BM, periosteum, and umbilical cord blood derived cells failed to generate muscle cells or myofibers after 4 weeks. In contrast, human muscle-derived CD146+ cells were distributed across the muscle interstitium and below the basement membrane on the surface of myofibers, demonstrating that they acquired a satellite cell-like position. In addition, these transplanted cells contributed to integrated myofibers detected by expression of human Dystrophin 2 and Spectrin. Human muscle CD146+ cells were transduced to express GFP, suspended in matrigel and injected into the epifascial space of the back of immunocompromised mice. Following a 3 week period, the samples were harvested and shown to form an extensive network of myotubes (that expressed human-specific myogenic markers desmin and myosin heavy chain), band also stratified myofibers. No myogenic differentiation was observed when using non-muscle derived CD146+ cells. Using RNA-seq, *in vitro*, and *in vivo* approaches, Sacchetti et al demonstrated that while BM CD146+ MSCs are inherently geared to generate bone and haematopoiesis stroma, their muscle counterparts are not. Instead, muscle CD146+ MSCs carrying an identical immunophenotype are not skeletogenic but inherently myogenic (Fig. 4). These observations were also consistent with other studies where *in vivo* transplants of BM cells form bone while adipose-derived cells and skin fibroblasts do not [72, 73]. The molecular drivers were not investigated by Sacchetti et al but it is plausible that an epigenetic blueprint is active in these cells conferring their myogenic identity even while they are perivascular. Using the studies as focal points for additional illustrative examples of

similar work, it is evident that there is a wealth of experimental evidence in the literature demonstrating that MSCs and their precursors are pre-programmed. Such evidence demonstrates that mechanisms are in place that define pericyte identity. Independent studies have demonstrated that MSCs and their precursors, irrespective of their residing tissue, are not identical populations (albeit with very similar immunophenotypes). These MSCs and their perivascular precursors have broad differentiation capabilities (as shown by *in vitro* assays), therefore it would be a biological necessity that molecular checkpoints are placed to prevent non-specific differentiation *in vivo.* Progressive deterioration of the robustness of these molecular mechanisms could be an underlying cause of why during aging (in humans and experimental animals) pericytes increasingly contribute to scarring and/or fibrosis as opposed to maintaining tissue homeostasis via regeneration or repair mechanisms [46, 49, 74].

A number of unanswered questions remain. Seeing that pericytes are innately heterogeneous, even within the same tissue, it is unclear if cell surface marker expression might possibly correlate with functional heterogeneity. Population dynamics need to be better understood to elucidate the innate tissue architecture and how various pericyte subpopulations could be differentially contributing to observed characteristics. To this end, single cell RNA-seq has been utilised in a number of organs including bone marrow, lung and the nervous system [75-77] , with Zeisel et al (2018) identifying 3 transcriptomically distinct populations of pericytes in brain[77].

Discussion and future considerations

To some extent, the “MSC” field has recognised that MSCs *in vitro* derived from a range of tissues are inherently different. These differences range from their colony forming efficiencies (CFU-Fs being a classical test of stemness) [78], their bias towards certain mesodermal lineages [71], or even their ability to regulate the immune system [79]. While *in vivo* research aimed at characterising epigenetic mechanisms of MSCs is limited, they are supported by observations in MSCs *in vitro*. Spontaneous differentiation of human bone marrow MSCs during culture expansion has been documented, not surprisingly this coincided with the expression of genes involved in osteogenesis (such as *Runx2*) coupled with repression of genes

involved in self-renewal and proliferation [80]. The importance of maintaining an appropriate epigenetic fingerprint is being recognised when taking into account that a number of MSC derived tumours are a consequence of epigenetic deregulation [81, 82]. It is therefore apparent that a more in-depth exploration of the epigenome of these cells, is needed to understand how they behave. In addition, single cell RNAseq studies are required as a tool to better design lineage tracing strategies to aid in identifying if a truly multipotent MSC exists *in vivo*. As an illustrative example, if adipose MSCs are programmed to make adipocytes then any potential therapeutic applications of these cells outside making adipose must ensure that the epigenetic programmes are fully erased as this will hinder their functional contribution. The importance of understanding more of these predetermined anatomical epigenetic programmes lies in the use of heterotypic MSCs in regenerative medicine approaches. Evidence presented here demonstrates that pericytes carry an intrinsic "memory" in the form of an epigenetic program that can form the basis of restricting their differentiation potential in vivo. Current approaches of in vitro stimulation presumably attempt to progressively modify the epigenome of these cells to allow differentiation into inappropriate (for their anatomical location) lineages. To the best of our knowledge this reprogramming is inefficient as composition of mineral produced by MSCs sourced from different tissues varies widely and so does the expression of defining molecular markers and gene expression patterns [83-85].The difficulty of efficiently differentiating MSCs in vitro, their degree of heterogeneity, and the inability to effectively demonstrate multipotency by lineage tracing raises the question if a truly multipotent MSC does exist in vivo.

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Figure Legends

Graphical Abstract: Pericytes isolated from stroma of multiple tissues can give rise to "MSCs" in vivo and in vitro. These MSCs have been shown in vivo to differentiate into specific mesenchymal derivatives appropriate to their anatomical location due to a prevailing epigenetic program limiting their differentiation potential. This is also the case in vitro when stimulating cocktails are not added to the base medium, as indicated by spontaneous differentiation of these cells. An in vivo, multipotent MSC that can give rise to multiple mature mesenchymal derivatives has not been confirmed.

Figure 1. Pericyte response to injury (or repair) *in vivo*. As part of a physiological homeostatic response, or an activation brought about by tissue injury, pericytes resting on the underlying vasculature **(A)** detach from the blood vessel walls and become bona fide mesenchymal stem cells **(B).** These MSCs have the ability to proliferate and give rise to more progenitors **(B).** These pMSCs can proliferate and move to an area of injury and subsequently differentiate into cells (tissue-specific) that facilitate repair **(C).** When pericytes are isolated and subsequently expanded in vitro, they can give rise to multiple mesodermal derivatives depending on the supplements they are supplemented with **(D).** *Adapted from Yianni & Sharpe (2019)* [57]

Figure 2. Isolation of pericytes for next generation sequencing experiments. Utilising the *XLacZ4* transgenic mouse line, pericytes expressing β-galactosidase can be isolated from incisor dental pulp and bone marrow **(A)**. Following enzymatic dissociation of incisor pulp and aspiration of bone marrow, β-galactosidase expressing pericytes can be FACS isolated as a purified population. These cells were then processed downstream for histone ChIP-seq and bulk RNA-seq **(B).**

Figure 3. Epigenetic landscapes restrict lineage identity in MSCs. Pericytes resident in bone marrow have a histone landscape that is permissive to the expression of early (Runx2 & Osx) and late (Ibsp) osteogenic genes. Genes of inappropriate cell fates are enriched for repressive heterochromatin. These include adipogenic (*Cebpa, Cebpb*), chondrogenic (*Sox9, Osr1, Osr2*) and myogenic (*Myf5, Pax3, Myod1*) cell fate specifying and/or marker genes **(A).**

Isolating and expanding these cells in vitro without the presence of any stimulant, results in upregulation of only osteogenic lineage appropriate genes such as *Runx2*.

Figure 4. Lineage restriction in MSCs sourced from multiple sources. Cells with a mesenchymal phenotype were FACS isolated from skeletal muscle, umbilical chord blood, and bone marrow based on expression of CD146, and lacking expression of CD45 and CD34. These cells were then culture expanded and assayed for their differentiation potential **(A).** Cells isolated from skeletal muscle could only give rise to myoblasts yet were unable to differentiate into chondrocytes, stromal cells or osteoblasts **(B).** Cells isolated from bone marrow could give rise to haematopoietic supporting stromal cells and osteoblasts but failed to differentiate into myoblasts, chondrocytes or adipocytes **(C).** *Adapted from Yianni & Sharpe (2019)* [57]

Figure 5. Epigenetic blueprints enforcing cell type specific transcriptomes. Pericytes in vivo, carry an epigenetic program which will guide their downstream differentiation potential into a lineage appropriate for their organ of residence **(A)**. Isolating pericytes and expanding them in vitro using a tailored stimulating cocktail will trigger a re-arrangement of the epigenetic landscape allowing expression of lineage specific genes corresponding to a different lineage **(B).** In vivo, pericytes carry a distinct (for every organ) epigenetic fingerprint specifying the cell type they will generate which is appropriate to their tissue of residence **(C).**

Supplementary Figure 1. Transcriptomic profiling of pericytes shows lineage priming prior to differentiation. Perivascular cells were isolated from incisor and skeletal muscle respectively. For incisor this was done using the XLacz4 mouse. For muscle this was done using FACS to isolate CD146+/CD34-/CD45- cells **(A).** Fresh incisor pericytes were then subjected to ChIP-seq RNA-seq, whereas cultured muscle pericytes expanded in basal media prior to RNA-seq and differential expression testing **(B).** Pericytes from incisor pulp carry a permissive chromatin landscape (euchromatin – H3K4me3 enriched) at the promoters of classically defined odontogenic genes such as *Dspp*. In addition to an odontogenic transcription program reminiscent of what you would expect mature odontoblast to

express. Conversely gene enrichment analysis from muscle pericytes revealed expression of genes regulating muscle development, muscle contraction and energy metabolism **(C)**.

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Authors Statement

Both authors contributed to the writing of this review.

Highlights

How epigenome modifications regulate mesenchymal stem cell behaviour in vivo

How transcriptome and epigenome differences between stem cell precursors from two different tissues predict in vivo cell differentaition

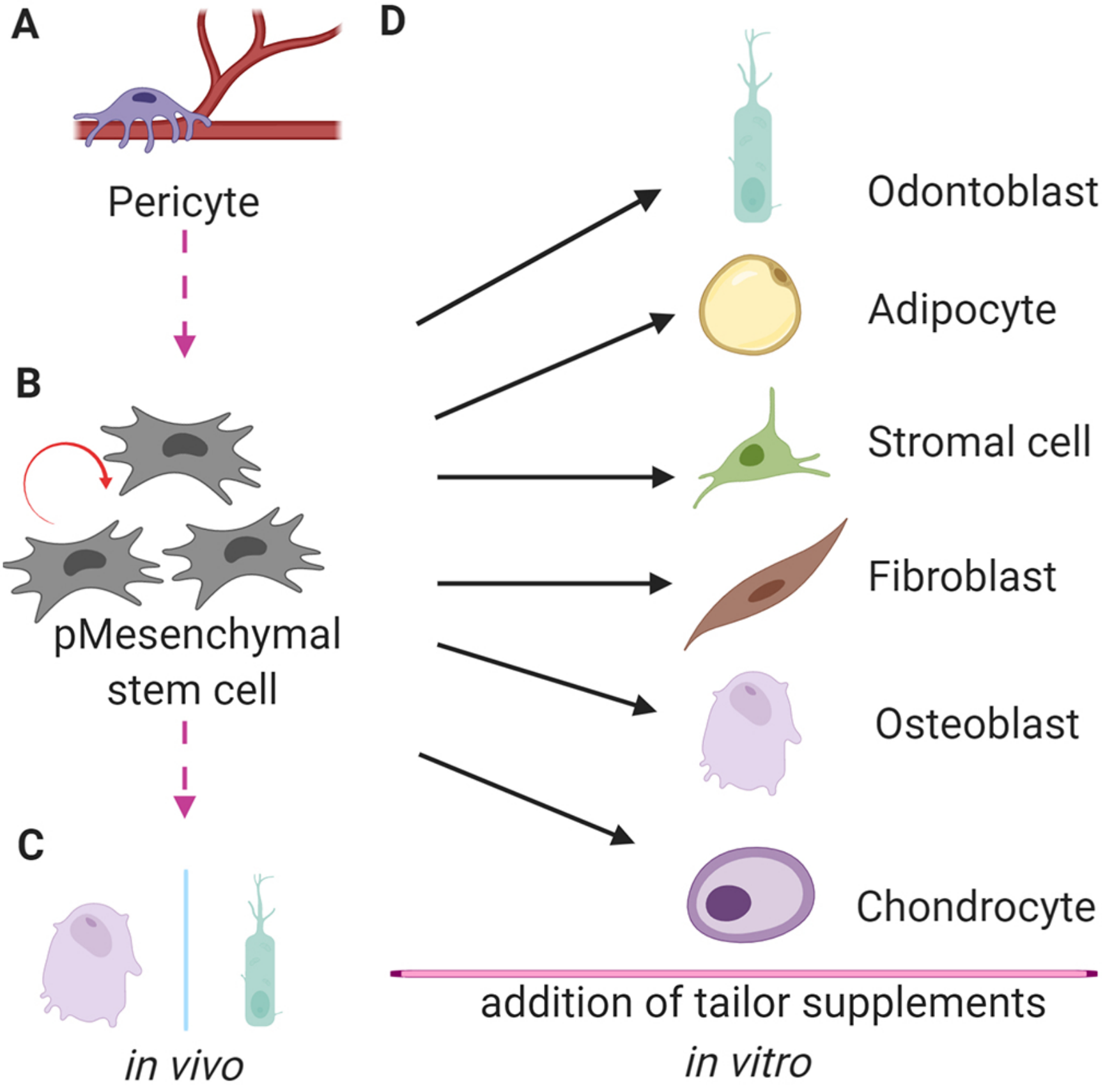
A
Pericyte
B
pMesenchymal
stem cell
C
in vivo
D
Odontoblast
Adipocyte
Stromal cell
Fibroblast
Osteoblast
Chondrocyte
addition of tailor supplements
in vitro

Figure 1

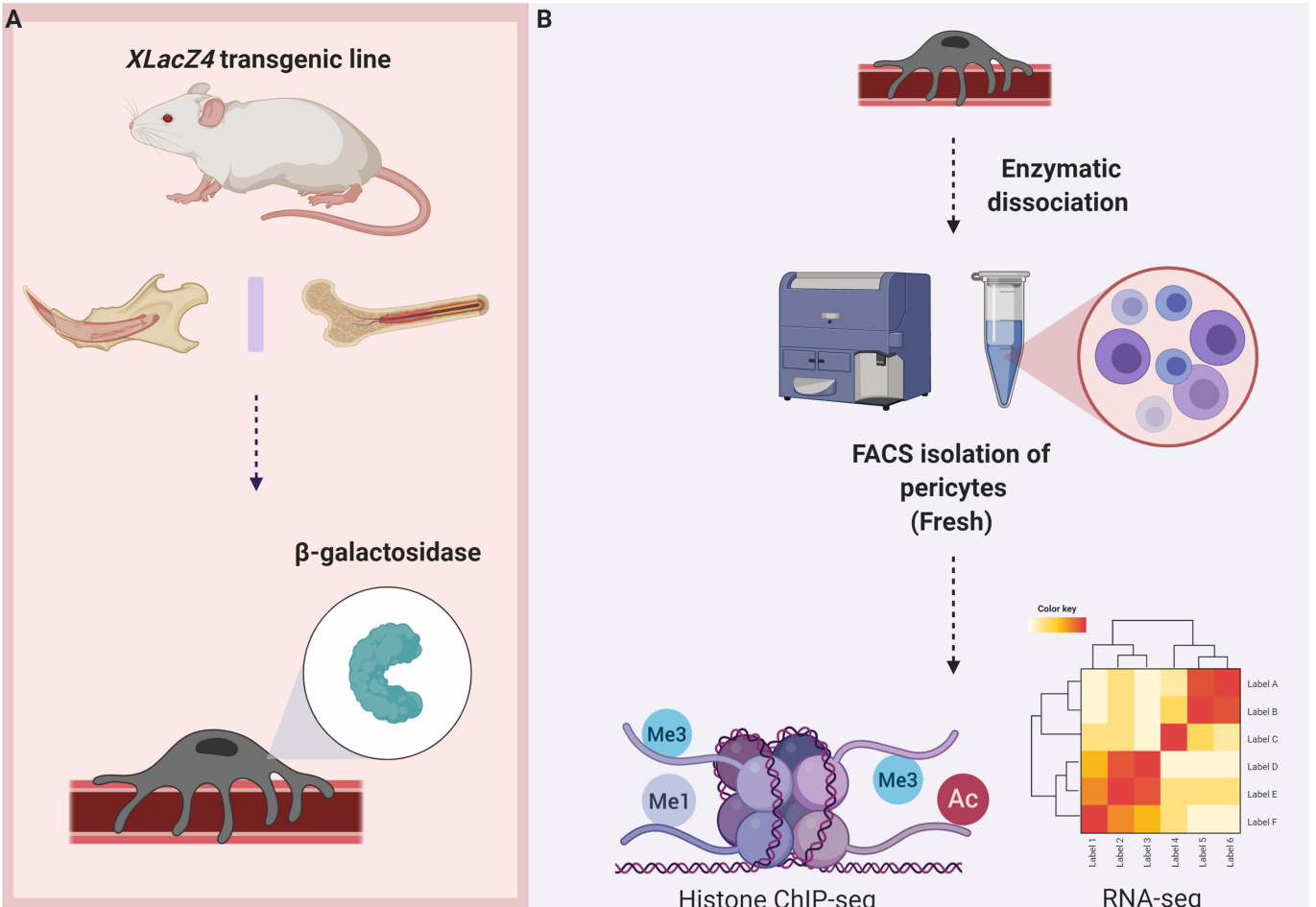
A
XLacZ4 transgenic line
β-galactosidase
B
Enzymatic dissociation
FACS isolation of pericytes (Fresh)
Me3
Me1
Me3
Ac
Histone ChIP-seq
Color key
Label A
Label B
Label C
Label D
Label E
Label F
Label 1
Label 2
Label 3
Label 4
Label 5
Label 6
RNA-seq

Figure 2

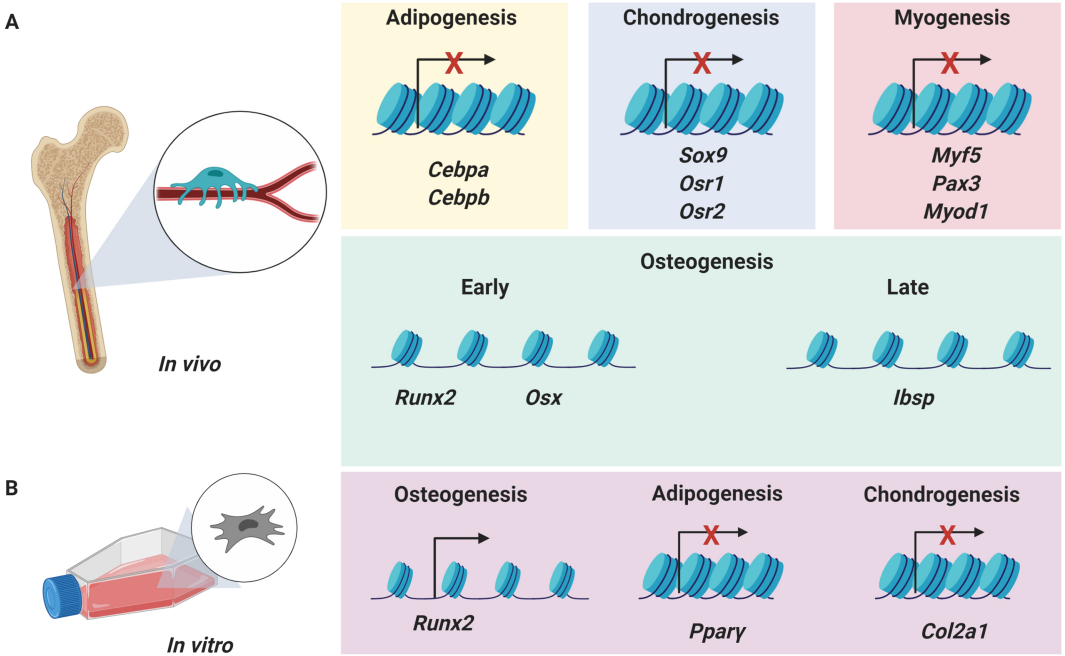
A
Adipogenesis
Cebpa
Cebpb
Chondrogenesis
Sox9
Osr1
Osr2
Myogenesis
Myf5
Pax3
Myod1
Osteogenesis
Early
Runx2
Osx
Late
Ibsp
In vivo
B
Osteogenesis
Runx2
Adipogenesis
Pparγ
Chondrogenesis
Col2a1
In vitro

Figure 3

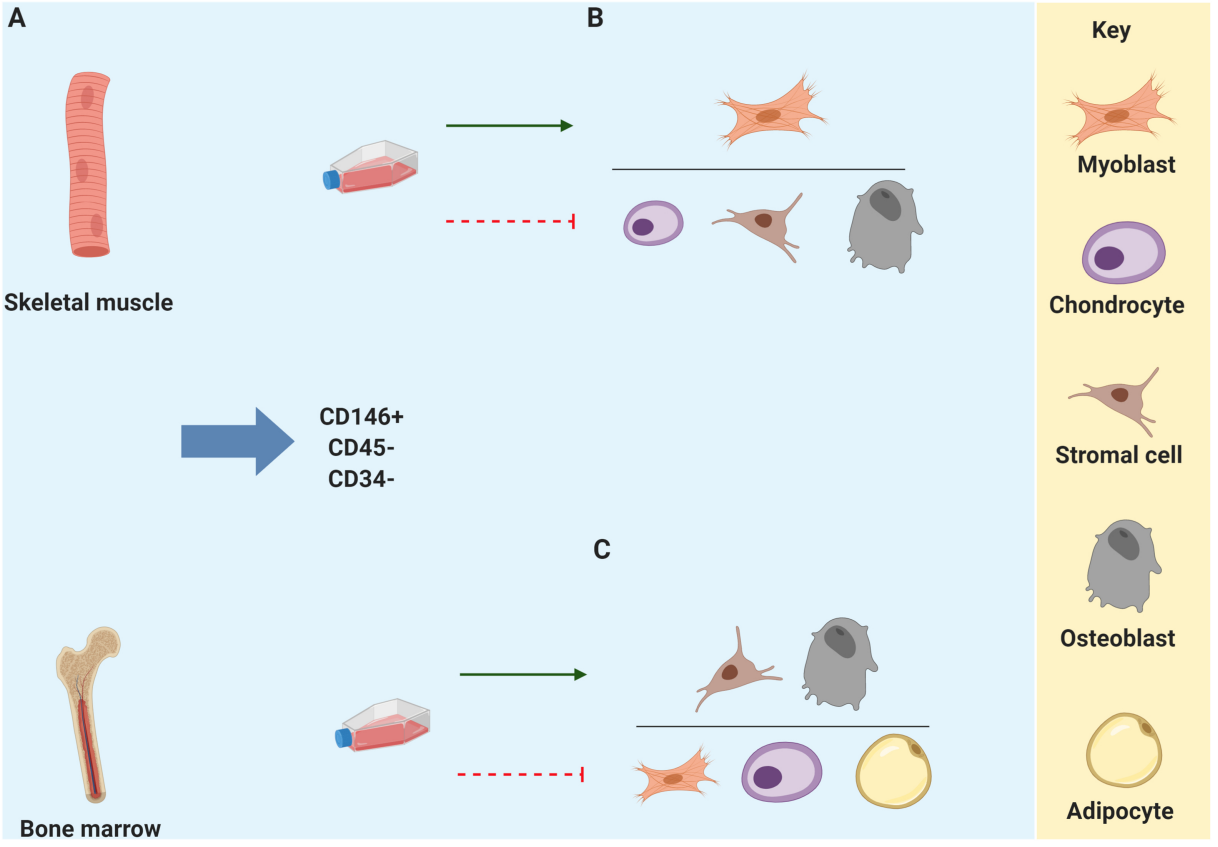
A
B
Key
Myoblast
Skeletal muscle
Chondrocyte
CD146+
CD45-
CD34-
Stromal cell
C
Osteoblast
Bone marrow
Adipocyte

Figure 4

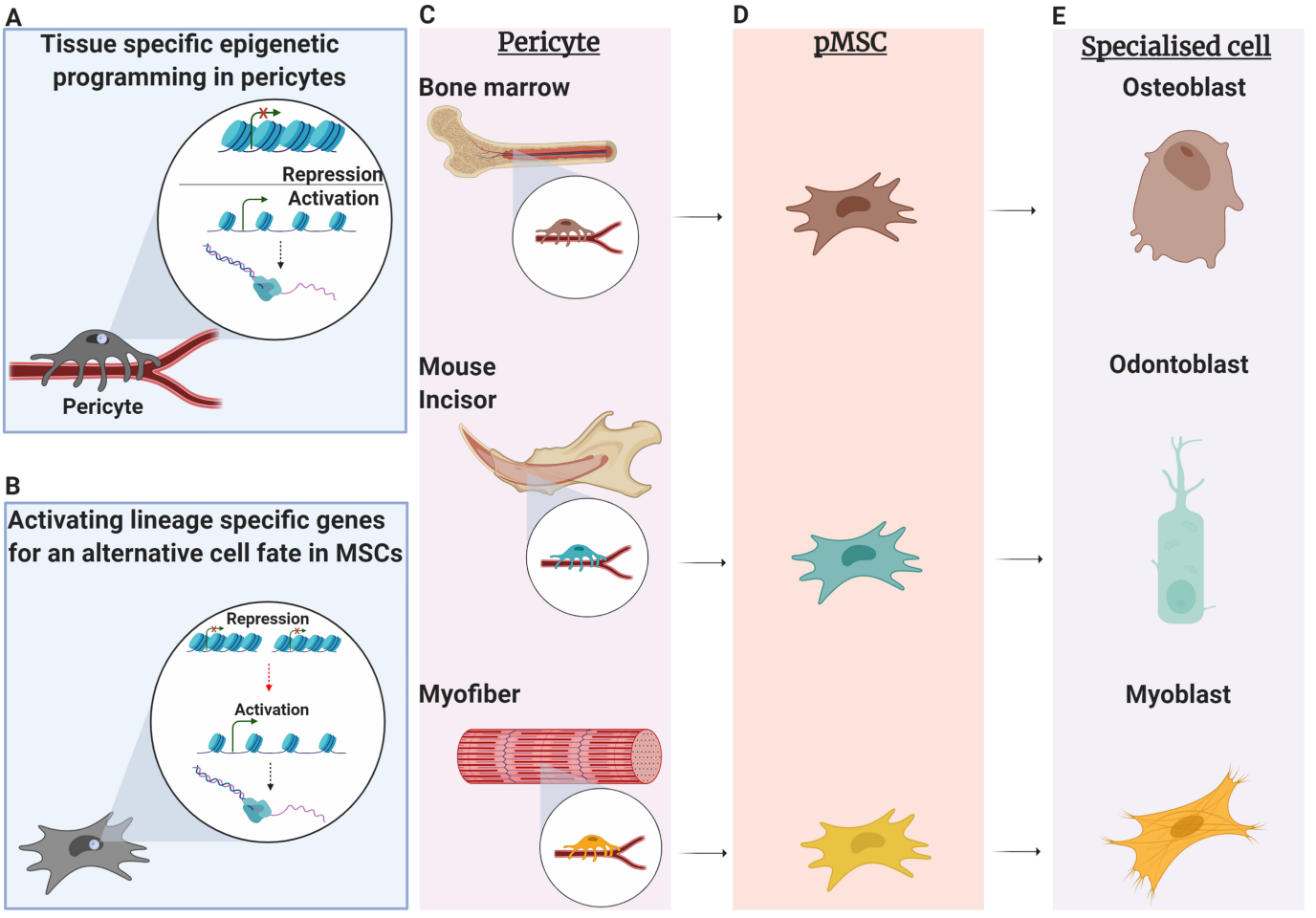
A
Tissue specific epigenetic programming in pericytes
Repression
Activation
Pericyte
B
Activating lineage specific genes for an alternative cell fate in MSCs
Repression
Activation
C
Pericyte
Bone marrow
Mouse Incisor
Myofiber
D
pMSC
E
Specialised cell
Osteoblast
Odontoblast
Myoblast

Figure 5

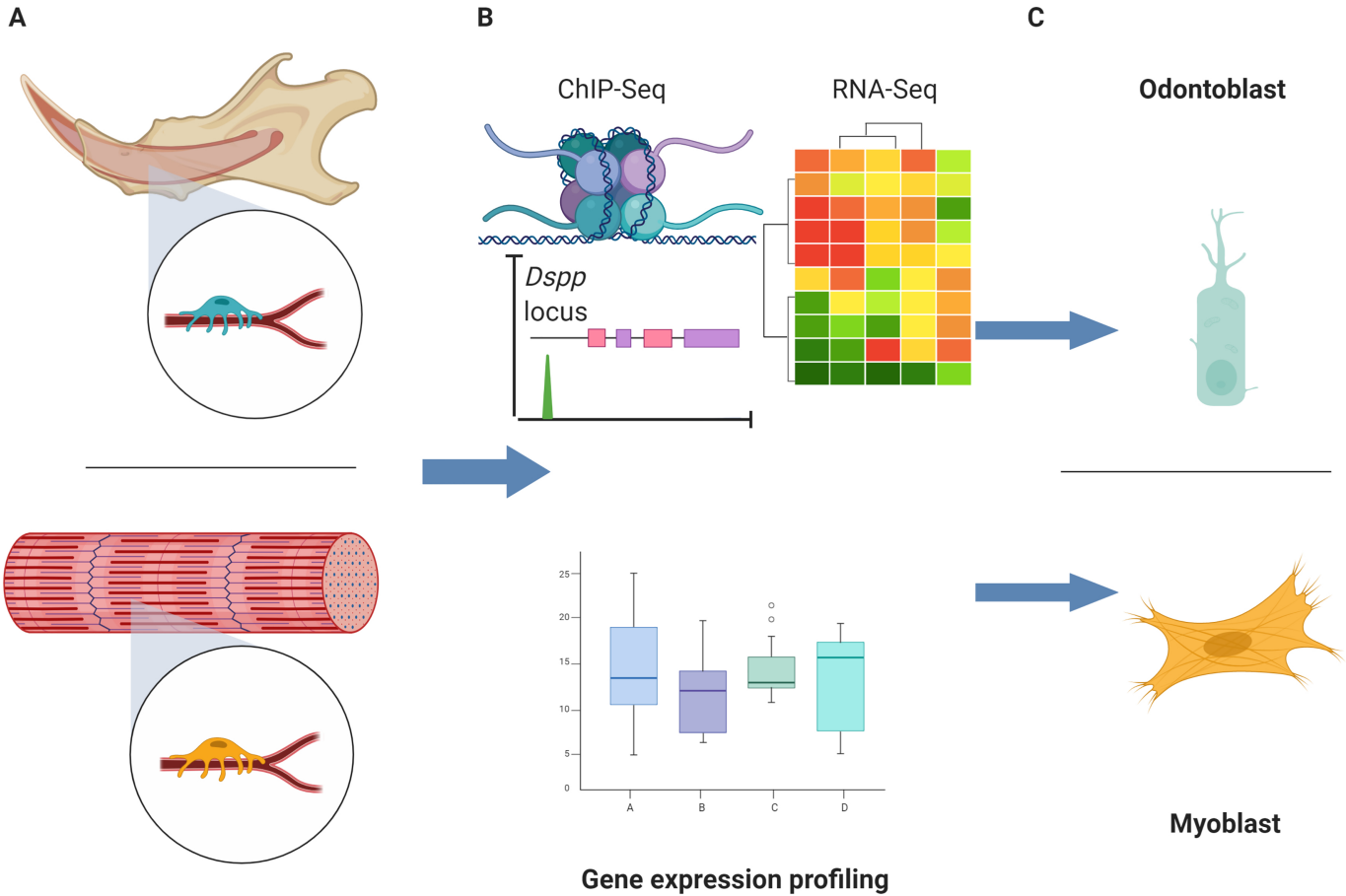
A
B
C
ChIP-Seq
RNA-Seq
Odontoblast
Dspp
locus
25
20
15
10
5
0
A
B
C
D
Gene expression profiling
Myoblast

Figure 6