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The spatiotemporal expression patterns of MSC-associated markers contribute to the identification of progenitor subpopulations in developing limbs

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ABSTRACT During limb development, skeletal tissues differentiate from their progenitor cells in an orchestrated manner. Mesenchymal stromal cells (MSCs), which are considered to be adult undifferentiated/progenitor cells, have traditionally been identified by the expression of MSC-associated markers (MSC-am) and their differentiation capacities. However, although MSCs have been isolated from bone marrow and a variety of adult tissues, their developmental origin is poorly understood. Remarkably, adult MSCs share similar differentiation characteristics with limb progenitors. Here, we determined the expression patterns of common MSC-am throughout mouse hindlimb development. Our results demonstrate that MSC-am expression is not restricted to undifferentiated cells *in vivo*. Results from the analysis of MSC-am spatiotemporal expression in the embryonic hindlimb allowed us to propose five subpopulations which represent all limb tissues that potentially correspond to progenitor cells for each lineage. This work contributes to the understanding of MSC-am expression dynamics throughout development and underlines the importance of considering their expression patterns in future MSC studies of the limb.

KEY WORDS: *MSC, limb progenitor cell, limb subpopulation, MSC marker*

Limb development originates from a bud of mesodermal cells, which are enveloped by a layer of ectodermal cells. At the distal-most region of the limb bud, a thickening of the ectoderm is formed, which is known as the apical ectodermal ridge (AER). Limb bud mesodermal cells are the progenitors from which adult limb tissues develop (Zeller *et al.*, 2009). In the mouse, the limb bud becomes distinguishable at the E9.5 stage. Skeletal tissue progenitors, dermis, tendons, and ligaments are derived from the lateral plate mesoderm (Johnson and Tabin, 1997). In contrast, muscle progenitor cells migrate from adjacent somites to limb buds at the E10.5 stage (Chevallier *et al.*, 1977). The AER releases signals that maintain the undifferentiated state of cell progenitors. Once these signals stop being received, cells commit to the chondrogenic lineage and initiate skeletal development (ten Berge *et al.*, 2008). Skeletal limb development occurs sequentially from the proximal to the distal course by initiating mesoderm condensation (Marín-Llera *et al.*, 2019). Chondrogenesis starts at the E11.5 stage, which is evidenced by the presence of mesodermal condensations in the limb bud core. Later, a cartilage anlage prefigures the skeletal elements (Akiyama *et al.*, 2005). Digital rays are evident at the E12.5 stage (Cooper *et al.*, 2013). Ossification begins at the E13.5 and E14.5 stages when cartilage cells in the center of the most proximal skeletal elements become hypertrophic. At the same time as endochondral ossification, blood vessel invasion occurs, and the mineralization process is favored (Mackie *et al.*, 2008). Following E14.5, the primary ossification center is formed. Here, most hypertrophic chondrocytes die by apoptosis, the first signs of mineralization in skeletal elements appear, and the periosteum-residing osteoblasts produce bone extracellular matrix (Kozhemya-

Abbreviations used in this paper: AER, apical ectodermal ridge; BM, bone marrow; MSC, mesenchymal stromal cell; MSC-am, mesenchymal stromal cell-associated markers.

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kina *et al.*, 2015). Subsequently, proliferating chondrocytes that reside in the bone metaphyseal region contribute to the formation of the growth plate, allowing bone elongation (Newton *et al.*, 2019).

Concomitantly with skeletal tissue formation, muscle development takes place. At the E10.5 stage, myoblasts migrate from the dermomyotome region of the somites towards the limb bud (Tam, 1981). Once myogenic progenitors reach the limb buds, they initiate differentiation by reorganizing their cytoplasm and aligning themselves to form chain-like structures. At E12.5, myoblasts fuse, forming multinucleated muscle cells. In mice, muscle cells can be morphologically identified as early as the E12.5 stage (McPherron *et al.*, 1997). Although most progenitors differentiate into mature skeletal muscle, some progenitors remain undifferentiated throughout development to establish the muscle stem cell population known as satellite cells (Lepper and Fan, 2010).

MSCs reside in many adult tissues and exhibit the capacity to differentiate into osteocytes, chondrocytes, and adipocytes *in vitro*. MSCs might also play an important role in tissue renewal and immunomodulation processes (English, 2013). Interestingly, their differentiation potential coincides with most tissues present in a functional limb. In addition to other adult and fetal sources, MSCs have been identified in the bone marrow (BM) (Bianco, 2014). The criteria used to identify MSCs include plastic adherence, expression of the MSC-am CD73, CD90, and CD105, the lack of CD45, CD34, CD19, CD11b, CD79a, and HLA-DR, as well as multipotent differentiation capacity (Dominici *et al.*, 2006). Additionally, CD29, CD44, and Sca1 markers have been reported as common MSC-am in mice. Multiparametric analyses using these MSC-am have allowed the identification of MSC subpopulations in different tissues (Jones *et al.*, 2006; Morikawa *et al.*, 2009; Pinho *et al.*, 2013). However, most of these assays were performed after *in vitro* expansion rather than in freshly isolated cells (Bianco, 2014). Notably, cell culture modifies MSC-am expression, suggesting that isolation based on *in vitro* characteristics may not reflect the cell identity of *in vivo* resident MSC *p*opulations (Marín-Llera and Chimal-Monroy, 2018). Therefore, the markers heterogeneity of the MSCs and differential distribution across tissues may be due to the acquisition of the MSC-am after culture.

The detection of MSCs has been extensively studied in many tissue types. However, knowledge about the developmental origin of these cells is limited. For example, although adult MSCs in the BM have been established, their origin in the developing limb remains

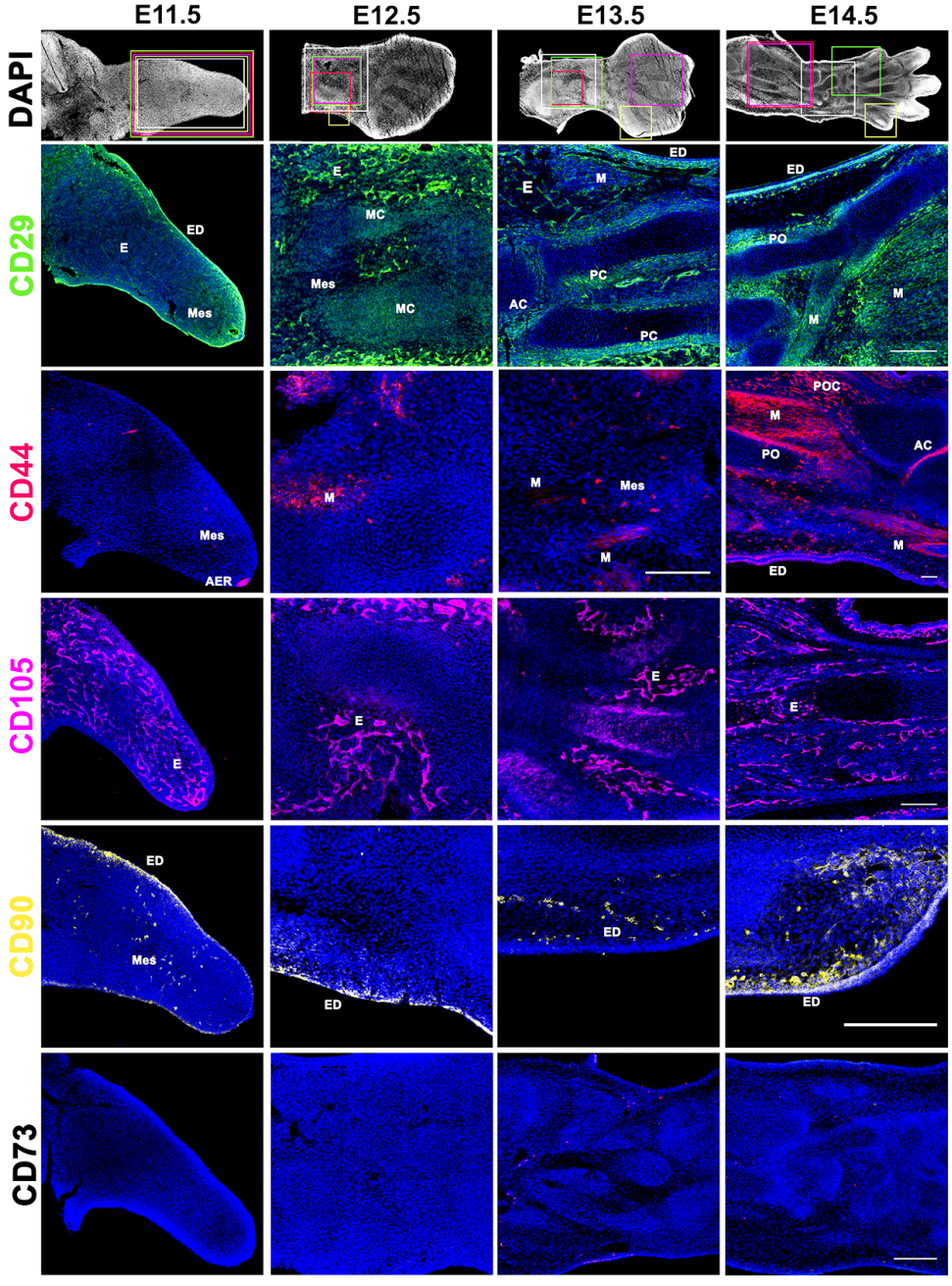

Fig. 1. Expression patterns of common mesenchymal stromal cell-associated markers (MSC-am) in mouse embryonic hindlimbs from stages E11.5 to E14.5. *Representative images of each limb developmental stage are shown in the superior panels. The magnification regions for CD29 (green), CD44 (red), CD105 (magenta), CD90 (yellow), and CD73 (white) are indicated with squares and represent the subsequent images. Abbreviations: AC, articular cartilage; AER, apical ectodermal ridge; E, endothelium; ED, ectoderm/dermis; M, muscle; MC, mesodermal condensations; Mes, mesenchyme; PC, perichondrium; PO, periosteum; POC, primary ossification center. Nuclei are stained with DAPI (blue). The scale bar is set at 200 μm and is representative of each marker.*

TABLE 1

LOCALIZATION OF INDIVIDUAL MSC-ASSOCIATED MARKERS ORGANIZED BY DEVELOPMENTAL STAGE

Marker	E11.5	E12.5	E13.5	E14.5	E15.5	E16.5	E17.5	E18.5
CD29	Undifferentiated mesoderm, endothelium, and ectoderm	Undifferentiated mesoderm, mesodermal condensations, endothelium and ectoderm	Mesodermal condensations, muscle, perichondrium, articular cartilage, dermis, Endothelium and ectoderm	Muscle, periosteum, articular cartilage, dermis, hair bud, endothelium, and ectoderm				
CD44	AER and few undifferentiated mesodermal cells	AER, few undifferentiated mesodermal cells, and muscle	Few undifferentiated mesodermal cells and muscle	Muscle, primary ossification center, articular cartilage, periosteum, and ectoderm			Muscle, primary ossification center, articular cartilage, periosteum, ectoderm, placodes and hair buds	
CD105	Endothelium					Endothelium and articular cartilage		
CD90	Ectoderm and few undifferentiated mesodermal cells	Ectoderm and dermis					Ectoderm and hair buds	
CD73	Expression not detected							

unknown. Recently, the ontogeny of two MSC subpopulations from adult BM and growth plates was analyzed in embryonic limbs by flow cytometry (Nusspaumer *et al*., 2017). However, this study did not determine the expression patterns of MSC-am in embryonic limbs. Therefore, there is no evidence that the subpopulations identified in adult tissues correspond to embryonic populations.

In the present study, we evaluated the spatiotemporal expression patterns of the MSC-am CD29 (Integrin-β1), CD44 (Hyaluronate receptor), CD105 (Endoglin), CD90 (THY1), and CD73 (ecto-5'-nucleotidase) throughout mouse limb development. The spatiotemporal patterns revealed that the evaluated MSC-am were not restricted to undifferentiated limb cells. Interestingly, the MSC-am exhibited similar expression patterns in some embryonic limb tissues. Based on this observation, in the present study we give insights into the identification of five subpopulations that potentially correspond to progenitor cells that originate from the limb tissues throughout development.

Results

Analysis of common MSC-am expression patterns in mouse hindlimbs during development

The criteria used to identify MSCs were established based on *in vitro* cell culture data, not *in vivo* data (Dominici *et al*., 2006). Here, we aimed to evaluate the spatiotemporal patterns of common MSC-am, such as CD29, CD44, CD105, CD90, and CD73, in hindlimbs from stages E11.5 to E18.5 (Fig. 1, Fig. 2, Sup. 1 and Table 1). First, we observed that CD29 expression was broadly distributed during limb development, including the mesodermal tissue during the E11.5 and E12.5 stages (Fig. 1). Posteriorly, CD29 is localized in mesodermal condensations in E12.5 and E13.5 (Fig. 1, Sup. 1A). Also, from E13.5 to E18.5, the expression of CD29 was observed in several tissues such as muscle, perichondrium, periosteum, articular cartilage, dermis, placodes, and hair buds (Fig. 1, Fig. 2 and Sup. 1A). Endothelial and ectodermal tissues were also positive for CD29 during all evaluated stages (Fig. 1, Fig. 2, and Sup. 1A).

At the early developmental stages, from E11.5 to E13.5, CD44 was expressed in the AER and some undifferentiated mesodermal cells (Fig. 1 and Sup. 1B). In the muscle tissue, CD44 was detected from stages E12.5 through E18.5 (Fig. 1 and Fig. 2). CD44 expression was also observed in the primary ossification center, articular cartilage, periosteum, and ectoderm from stages E14.5 through E18.5 (Fig. 1, Fig. 2 and Sup. 1B). Moreover, hair buds expressed CD44 at E17.5 and E18.5 (Fig. 2 and Sup. 1B).

In contrast, CD105 expression was detected in the endothelial cells during all analyzed developmental stages (Fig. 1 and Fig. 2). CD105 expression was also observed in the articular cartilage from stages E15.5 to E18.5 (Fig. 2). Only a few undifferentiated mesodermal cells were positive for CD90 during E11.5 (Fig. 1). Furthermore, CD90 expression was localized and remained in the ectoderm, dermis, and placodes or hair buds from E11.5 through development (Fig. 1 and Fig. 2). Finally, CD73 expression was not detected in any of the developmental stages analyzed (Fig. 1 and 2). These results demonstrated that MSC-am expression is dynamic during limb development and is not restricted to undifferentiated cells *in vivo.* Notably, not all MSC-am were expressed in the same cells or tissues in time and space.

Identification of prospective progenitor subpopulations in the embryonic limb based on MSC-am expression patterns

Our observations suggest that at only some developmental stages do limb tissues co-express MSC-am. On this basis, we proposed the presence of five subpopulations that could be used to identify precursor or committed cells for different tissues throughout embryonic limb development (Fig. 3 and Table 2). For all tissues and stages, the expression patterns of the MSC-am CD29, CD44, CD105, and CD90 were considered. Each subpopulation was distinguished and immunophenotyped according to the type/s of MSC-am they expressed. Four of the five subpopulations shared the expression of CD29. The first subpopulation corresponded to the $CD29^{+}CD44^{+}CD90^{+}$ cells found in the undifferentiated mesoderm and ectoderm in the E11.5 stage. This subpopulation was later restricted to the ectoderm, dermis, and hair buds starting at E12.5 and continuing throughout limb development. The second subpopulation, $CD29^{+}CD105^{+}$ cells, was detected in endothelial tissue throughout all evaluated limb developmental stages. The third subpopulation, $CD29^{+}CD44^{+}$ cells, was observed in the muscle from stages E13.5 to E18.5 and in the periosteum from E14.5 to E18.5. The fourth subpopulation, $CD29^{+}CD44^{+}CD105^{+}$ cells, was first detected at E15.5 in the articular cartilage, where they remained until the E18.5 stage. Finally, the fifth cell subpopulation, $CD44^{+}$ cells, was detected in the primary ossification center from E14.5 to E18.5 (Fig. 3 and Table 2).

Discussion

Adult MSCs are recognized by their differentiation potential and role in regenerative medicine (Kolf *et al*., 2007; Valtieri and Sorrentino, 2008). However, the developmental origin of adult MSCs

is poorly understood. Most MSC studies have been based on *in vitro* analyses, which may not accurately represent *in vivo* MSC populations. In this work, we evaluated the expression patterns of common MSC-am in the developing mouse hindlimb. We found a differential distribution of all markers among limb tissues at different developmental stages. Notably, we did not find co-expression of all MSC-am as described for *in vitro* MSC populations (Bianco, 2014; Dominici *et al*., 2006; Kolf *et al*., 2007).

Jiao *et al.,* reported that human embryonic limb cells from Carnegie stage 16, equivalent to E13.5 in mouse, express the markers CD13, CD29, CD90, CD105, and CD106 (Jiao *et al*., 2012). However, this analysis was performed after *in vitro* expansion. Contrary to Jiao et. al findings, we found that endothelial cells expressed CD29 and CD105 *in vivo*. At the same time, CD90 is restricted to the ectoderm, dermis, and prospective hair buds at the E13.5 developmental stage. The present study results suggest that cells expressing MSC-am after *in vitro* expansion may not identify the same multipotent progenitor populations *in vivo*. As such, MSC-am expression after *in vitro* expansion does not follow the same trends as *in vivo* populations. Accordingly, the MSC-am immunophenotypes used to define multipotent cells *in vitro* might not correspond to *in vivo* MSCs.

Most studies are based on the identification and characterization of MSC subpopulations after *in vitro* expansion. Here, we identified five subpopulations in all limb developmental stages, which may correspond to a progenitor or committed cells for different limb tissues. The subpopulations identified in this study consider cell identity based on the expression pattern of each MSC-am. As development progresses, limb bud progenitor cells differentiate in an orchestrated and sequential manner. Notably, the limb subpopulations proposed herein encompass undifferentiated mesoderm, chondrogenic condensations, perichondrium, periosteum, articular cartilage, muscle, endothelium, and ectoderm derived tissues. Therefore, they represent all tissues in the developing limb, suggesting that they might correspond to cell progenitors. It is also possible that their phenotypes are maintained in adult tissues.

The function of MSC markers in MSCs is not thoroughly described. However, for limb cells, it has been demonstrated that CD29 deficiency results in chondrocyte proliferation and joint defects (Garciadiego-Cazares *et al*., 2004; Wu and Santoro, 1994). Additionally, CD29 has been found to play a role in regulating collagen synthesis in the skin (Gardner *et al*., 1999). Accordingly, we observed CD29 expression in

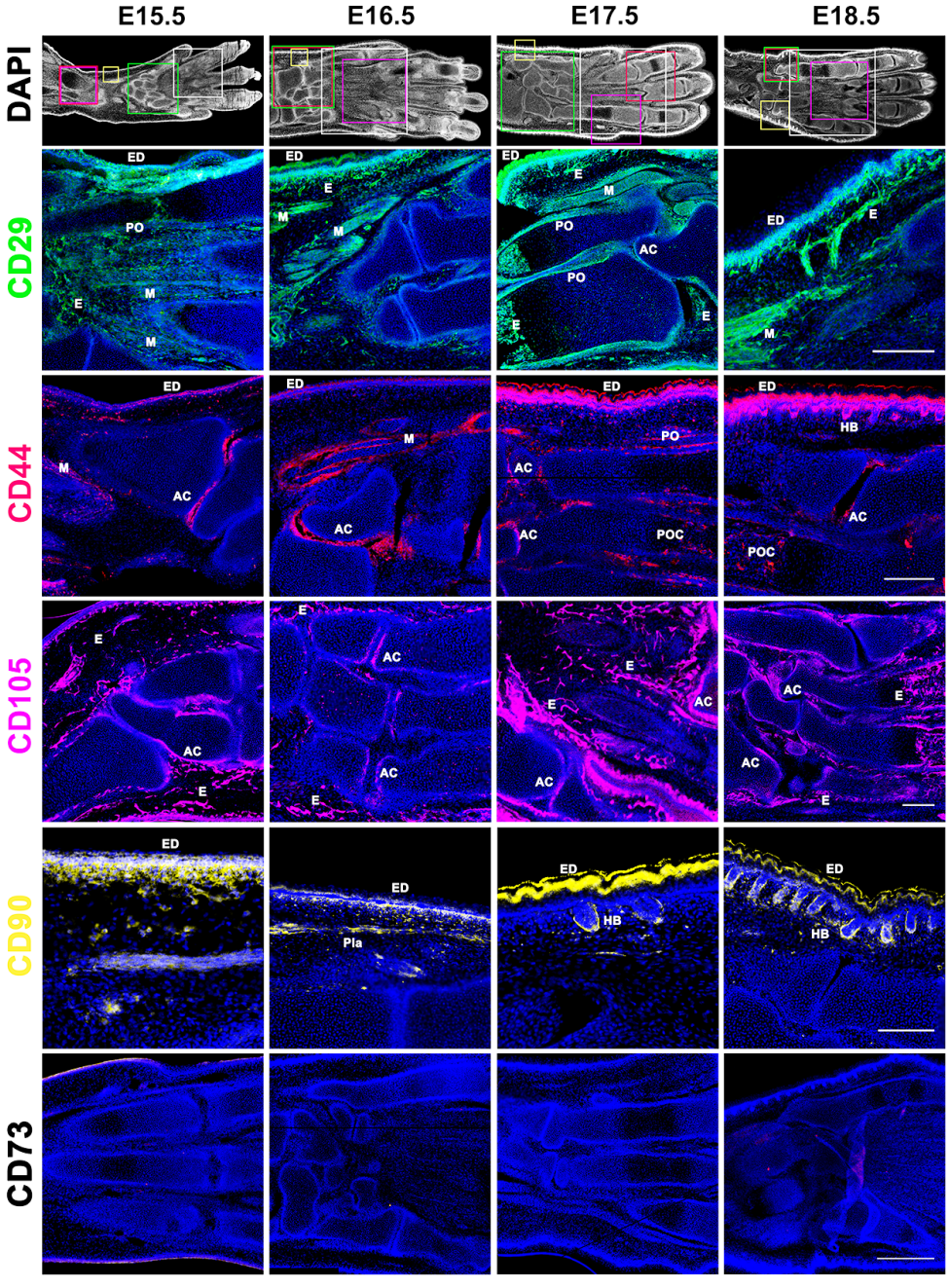

Fig. 2. Expression patterns of common mesenchymal stromal cell-associated markers (MSC-am) in mouse embryonic hindlimbs from stages E15.5 to E18.5. *Representative images of each limb developmental stage are shown in the superior panels. The magnification regions for CD29 (green), CD44 (red), CD105 (magenta), CD90 (yellow), and CD73 (white) are indicated with squares and represent the subsequent images. Abbreviations: AC, articular cartilage; E, endothelium; ED, ectoderm/dermis; HB, hair bud; M, muscle; Pla, placode; PO, periosteum; POC, primary ossification center. Nuclei are stained with DAPI (blue). The scale bar is set at 200 μm and is representative of each marker.*

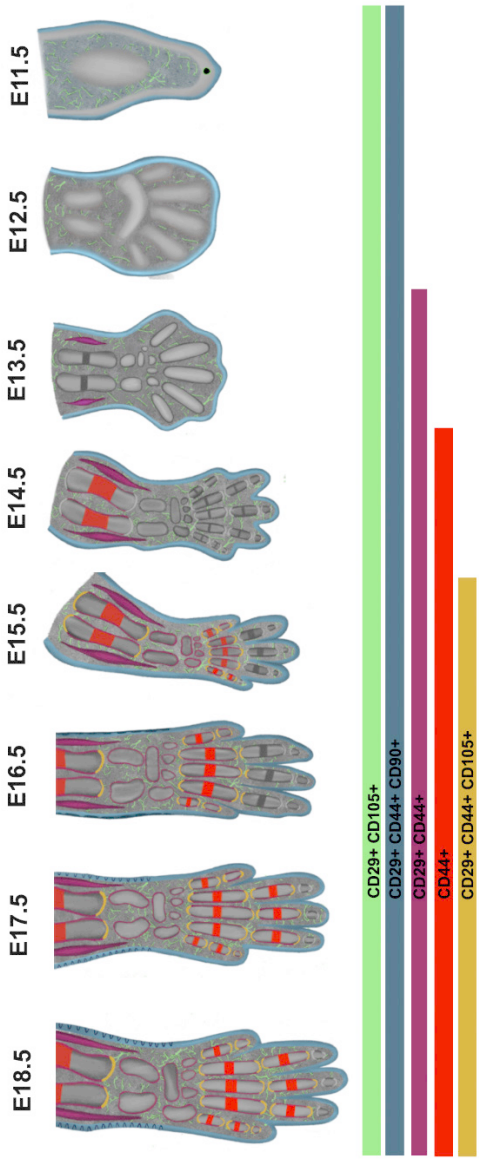

Fig. 3. Identified embryonic limb subpopulations based on the expression patterns of CD29, CD44, CD105, and CD90. *Schematic representation of mesodermal subpopulation in the hindlimb bud tissues based on combinatorial expression of evaluated MSC markers. Subpopulations are colored and categorized by limb tissue. Representation of CD29⁺ CD105⁺ (green), CD29⁺ CD44⁺ CD90⁺ (blue), CD29⁺ CD44⁺ (purple), CD44⁺ (red), CD29⁺ CD44⁺ CD105⁺ (yellow) subpopulations from stages 11.5 to 18.5. For tissue references see Table 2.*

chondrogenic condensations, articular cartilage, and dermis during limb development. Likewise, CD90 expression has been reported in prenatal murine skin and murine epidermal skin (Tschachler *et al.*, 1983). CD90 has been proposed as a regulator of cell-cell and cell-matrix interactions in several tissues, particularly fibroblasts related to wound healing (Rege and Hagood, 2006). Remarkably, our work is the first to evaluate the expression of CD90 during limb development.

Although CD105 is routinely used to identify adult endothelial tissues, it is also widely used to identify MSCs (Fonsatti *et al.*, 2001; Kolf *et al.*, 2007). The role of CD105 in development has been described in the endoglin knock-out mouse, which exhibits premature death around E11.5 due to vascular abnormalities (Li *et al.*, 1999). CD105 expression has also been detected in primary human articular chondrocytes of adults (Parker *et al.*, 2003). Here, we observed that CD105 identifies endothelial cells and articular cartilage in the early stages of development. Together these findings suggest that CD105 may be maintained in articular cartilage from embryonic to adult stages.

In skeletal muscle, CD44 has been described to play a role in progenitor migration and myoblast fusion (Gullberg *et al.*, 1998). Also, CD44 regulates cavitation during avian joint development (Dowthwaite *et al.*, 1998). In concordance with these previous findings, our results showed that CD44 is first expressed at the early stages of muscle and joint development.

CD73 expression has been observed in the ribs of E16.5 embryos and the epiphysis of the adult femur (Breitbach *et al.*, 2018). However, our results showed that in embryonic limbs, there was no expression of CD73 at any developmental stage. It has been reported that a CD73-positive cell subpopulation, included within the PαS limb population, has chondrogenic, osteogenic, and adipogenic potential (Nusspaumer *et al.*, 2017). However, this subpopulation was characterized in postnatal mouse bones, suggesting that CD73 expression arises postnatally.

Importantly, the cell function of MSC-am needs to be conceived in a combinatorial manner for each subpopulation at each developmental stage. The CD29⁺ CD105⁺ subpopulation is not mesodermal but an endothelial subpopulation. Hence, the function of CD29 is related to the integrity of blood vessels; the proper localization of VE-cadherin and cell-cell junction to avoid leaky vessels (Yamamoto et. al., 2015). Meanwhile, CD105 prevents apoptosis in this cell type (Li et. al., 2013).

CD29⁺ CD44⁺ CD90⁺ subpopulation transiently localizes in undifferentiated mesoderm at E11.5 but later is restricted to ectodermal tissue. The integrin-β1 blockade prevents the formation of cartilage nodules, reduce growth, increase apoptosis, and produces an abnormal organization of the actin cytoskeleton, implying that integrin-β1 is important for cartilage differentiation (Hirsch *et al.*, 1997; Shakibaei, 1998). Because mesodermal commitment and cell aggregation are the first steps of chondrogenesis (Marin-Llera et. al., 2019), at the E11.5 stage, CD29 expression in undifferentiated mesoderm could be implied with cartilage commitment, while CD44 regulates chondrocyte cell adhesion (Ishida *et al.*, 1997). Meanwhile, when the same subpopulation is expressed in ectodermal tissue, functions of the same markers could be associated with

TABLE 2

SUMMARY OF THE PROPOSED SUBPOPULATIONS BASED ON CO-EXPRESSION OF MSC-ASSOCIATED MARKERS THROUGHOUT LIMB DEVELOPMENT

Subpopulation	Stage	Tissue
CD29⁺ CD44⁺ CD90⁺ CD105⁻	11.5	Undifferentiated mesoderm
	11.5-18.5	Ectoderm, dermis and hair buds
CD29⁺ CD44⁺ CD105⁻ CD90⁻	13.5-18.5	Muscle
	14.5-18.5	Periosteum
CD29⁻ CD44⁺ CD105⁻ CD90⁻	14.5-18.5	Primary ossification center
CD29⁺ CD44⁺ CD105⁺ CD90⁻	15.5-18.5	Articular cartilage
CD29⁺ CD105⁺ CD44⁻ CD90⁻	11.5-18.5	Endothelium

Their complete immunophenotypes are presented.

keratinocyte adhesion, migration, hyaluronic acid (HA) production, healing, and skin homeostasis (Shatirishvili *et al*., 2016; Jiang and Rinkevich, 2018).

On the other hand, CD29$^+$ CD44$^+$ subpopulation emerges almost at the same developmental stage in muscle and perichondrium. In muscle, integrin-β1 (α7β1) is responsible for myofiber adhesion and cytoskeletal integrity, while CD44-expression plays a role in progenitor migration and myoblast fusion (Hodges *et al*., 1997; Gullberg *et al*., 1998). Correspondingly, both processes are highly coordinated for correct muscle development. Otherwise, the perichondrium plays a crucial role in bone elongation. Perichondral cells differentiate into osteocytes (Colnot *et al*., 2004), and it is reported that CD44 is expressed by osteocytes (Hughes *et al*., 1994). A described periosteum skeletal stem cell, a highly recruited population during bone repair, expresses CD29. The integrin-β1 mutant shows chondrodysplasia and delay in mineralization. At E14.5, the same developmental stage where we identified CD29+ CD44+ subpopulation arises, the length of the mutant humerus is significantly shorter (Aszodi *et al*., 2003). In this sense, emerging perichondral signals that control chondrocyte differentiation, bone elongation could be related to the expression of CD29 and CD44 markers.

In the primary ossification center, the zone where a skeletal element starts ossifying, a CD44$^+$ subpopulation is maintained through development. Several ligands of CD44, including HA, osteopontin (OPN), collagens, and matrix metalloproteinases (MMPs), have been reported (reviewed by Goodison *et al*., 1999). The presence of HA has been extensively related to angiogenesis in tumor cells. Accordingly, the angiogenic process is very active during bone formation. In addition, CD44 is activated by MMP9, leading to proteolytic cleavage of CD44, producing the intracytoplasmic domain CD44-ICD, which binds to RUNX2 to activate several bone differentiation genes (Okamoto *et al*., 2001; Miletti-González *et al*., 2012). This suggests that CD44 expression in the primary ossification center may contribute to the ossification process and bone differentiation.

Further, CD29$^+$ CD44$^+$ CD105$^+$ expression in joints was identified. The role of integrin-mediated adhesion and signaling in the physiology of articular cartilage is not fully understood. Nevertheless, CD29 inhibition results in ectopic joint formation, suggesting that integrin-β1 may be important for articular cartilage differentiation (Garciadiego-Cazares *et al*., 2004). HA is present in synovial fluid, playing an important role in protecting articular cartilage lubricating the joints due to its viscosity. HA protects joints from frictional damage forming a film between the cartilage surfaces (reviewed by Tamer, 2013). HA has been extensively described as an adhesive molecule. Nevertheless, since exogenous hyaluronan facilitates cell detachment, it has been proposed that HA is necessary for the synovial joint formation (Matsumoto, *et al*., 2009), in addition, to maintain the cavity's volume preventing secondary fusion across the joint space (Craig *et al*., 1990). On this basis, the localization pattern of CD44 in articular cartilage is reasonable, functioning as a HA binder and participating in forming the epithelial arrangement of the synovial lining layer. Finally, inhibition of TGF-β signaling in chondrocytes leads to chondrocyte terminal differentiation and the entire loss of articular chondrocytes (Shen *et al*., 2013). This observation suggests that CD105, as a TGFβ receptor, may contribute to maintaining the TGFβ signaling in articular cartilage through development.

On this basis, our results indicate that the *in vivo* function of MSC-am is related to tissue localization and may represent progenitor cells from the same tissue. For this reason, the expression of MSC-am *in vitro* may not reflect a specific cell function or potentiality in MSCs.

Our results provide evidence that the expression of MSC-am is not restricted to undifferentiated cells in the limbs of mice (and potentially other organisms). On the contrary, MSC-am were found in progenitor or committed cells and even in differentiated tissues, with a specific combinatorial expression at each developmental stage. Since the expression of MSC-am is not restricted to a unique set of multipotent undifferentiated cells *in vivo*, it is important to consider the expression patterns of MSC-am before isolating subpopulations after *in vitro* cell culture.

In conclusion, we demonstrated that MSC-am expression is found on committed and differentiated cells in the limb. Thus, MSC-am expression is not a trademark for undifferentiated cells, although they have been identified in multipotent cells *in vitro*. Importantly, the expression of MSC-am is dynamic during limb development. This highlights the importance of identifying subpopulations for each developmental stage by selecting an adequate cell marker combination.

Materials and Methods

Ethical statement

The protocol used in this research was reviewed and approved by the Institutional Review Board for the Care and Use of Laboratory Animals at the Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México (UNAM, Mexico City, Mexico).

Embryos

CD-1 pregnant mice at different gestational stages were euthanized by CO_2 asphyxiation. Embryos and fetuses from gestational days 11.5 to 18.5 were removed from the uterus and handled according to Marin-Llera and Chimal-Monroy, 2018. Hindlimbs were washed twice in chilled 1X PBS to eliminate blood and extraembryonic tissues and immediately processed for immunodetections. All the animals were obtained from the animal facility of the Instituto de Investigaciones Biomédicas, UNAM (Mexico City, Mexico).

Sample processing

Obtained hindlimbs of embryos from E11.5 through E18.5 were fixed in chilled Paraformaldehyde (PFA; Sigma-Aldrich, St Louis, MO, cat. no) 4% overnight at 4 °C. Fixed hindlimbs were washed in 1X PBS and dehydrated in an ascendant train of sucrose solution (10%-20%-30%) in PBS for 24h at 4°C. For samples exhibiting stages E15.5 through E18.5, hindlimbs were decalcified with 10%-EDTA solution in distilled water at 4 °C overnight. Once dehydrated and decalcified, hindlimbs were embedded in Tissue-Tek® (Sakura Europe, The Netherlands, cat.no 4583) inside embedding capsules (BEEM® Size 3; Hatfield, PA, cat. no. 69910) and frozen at -80 °C. Twenty-micrometer tissue sections were obtained using a cryostat (SLEE medical, model MEV, Germany). For samples from stages E11.5 and E12.5 (in Sup. 1), transversal sections were obtained. Coronal sections were performed on samples from stages E12.5 through E18.5. Cryosections were mounted on 2% gelatin-coated slides and placed in a vacuum chamber for 24h.

Immunofluorescence

TissueTek® embedded samples were washed three times with 1X PBS, and tissue was permeabilized with 0.3% Triton X-100 (VWR; England, UK; cat. no. M143) in 1X PBS for 15 min. Samples were incubated in pre-heated Immuno DNA Retriever Citrate® (BioSB, Santa Barbara, CA, cat. no. BSB0020) for 45 min at 65 °C. Samples were blocked in 2% of

Fetal Bovine Serum (FBS; Life Technologies, cat. Waltham, MA; cat. no. 26140) for two h at room temperature. Subsequently, the primary antibodies were used against CD29 (1:100; R&D Systems, Minneapolis, MN, cat. no. AF2405), CD44-PE (1:300; Biolegend, San Diego, CA, cat. no. 400607), CD105 (1:100; R&D Systems, cat. no. AF1320), CD73 (1:500; Abcam, cat. no. AB71822), and CD90 (1:100; R&D Systems, cat. no. AF7335). Primary antibodies were incubated at 4 °C overnight. Signals were detected using the following secondary antibodies: α-goat Alexa 488 (1:250; Invitrogen, Waltham, MA; cat. no. A11055) was used for CD29 and CD105, α-sheep Alexa 555 (1:250; Invitrogen, cat. no. A31373) was used for CD90, α-rabbit Alexa 555 (1:250; Invitrogen, cat. no. A31573) was used for CD73, and α-rat Alexa 555 (1:300; Invitrogen, cat. no. A21434) was used for CD44. Secondary antibodies were incubated for 2 h at room temperature and protected from light. Nuclei were counterstained with DAPI (1:500). Slides were washed and mounted with SlowFade™ Gold Antifade Mountant (Thermo Scientific, Waltham, MA, cat. no. S36937). Images were acquired using an Olympus BX51-WI epifluorescence vertical microscope equipped with a spinning disk unit (Olympus Corporation, Tokyo, Japan).

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Authors contributions

ASG-C performed the experiments. JC-M and JC-ML designed the study and analyzed the data. ASG-C participated in draft preparation. JC-M and JC M-L wrote the final version of the manuscript. All authors reviewed and approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.

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