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Article

Identification and characterization of chondrogenic progenitor cells in the fascia of postnatal skeletal muscle

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Intramuscular injection of bone morphogenetic proteins (BMPs) has been shown to induce ectopic bone formation. A chondrogenic phase is typically observed in this process, which suggests that there may exist a chondrogenic subpopulation of cells residing in skeletal muscle. Two prospective cell populations were isolated from rat skeletal muscle: fascia-derived cells (FDCs), extracted from gluteus maximus muscle fascia (epimysium) and muscle-derived cells (MDCs) isolated from the muscle body. Both populations were investigated for their cell surface marker profiles (flowcytometry analysis), proliferation rates as well as their myogenic and chondrogenic potentials. The majority of FDCs expressed mesenchymal stromal cell markers but not endothelial cell markers. FDCs underwent chondrogenic differentiation after BMP4 treatment *in vitro*, but not myogenic differentiation. Although MDCs showed chondrogenic potential, they expressed the myogenic cell marker desmin and readily underwent myogenic differentiation *in vitro*; however, the chondrogenic potential of the MDCs is confounded by the presence of FDC-like cells residing in the muscle perimysium and endomysium. To clarify the role of the muscle-derived myogenic cells in chondrogenesis, mixed pellets with varying ratios of FDCs and L6 myoblasts were formed and studied for chondrogenic potential. Our results indicated that the chondrogenic potential of the mixed pellets decreased with the increased ratio of myogenic cells to FDCs supporting the role of FDCs in chondrogenesis. Taken together, our results suggest that non-myogenic cells residing in the fascia of skeletal muscle have a strong chondrogenic potential and may represent a novel donor cell source for cartilage regeneration and repair.

Keywords: skeletal muscle, fascia, chondrocytes, cartilage

Introduction

Injection of bone morphogenetic proteins (BMPs) into skeletal muscle has been shown to induce ectopic endochondral bone formation (Kawai et al., 1994; Peng et al., 2005). This process comprises several sequential phases: inflammation, cartilage formation and resorption, and bone formation. However, it has not yet been determined whether the cells in the muscle, which can undergo chondrogenic and osteogenic differentiation, are the same or represent different cell populations. If chondrogenic and osteogenic progenitors are distinct, the cell population that undergoes cartilaginous cellular differentiation in skeletal muscle could provide an alternative cell source for cartilage repair.

It has been reported that skeletal muscle contains stem cells, which have multi-lineage differentiation potentials, including osteogenic, myogenic, and chondrogenic potentials (Qu-Petersen et al., 2002; Zheng et al., 2007; Crisan et al., 2008; Gharaibeh et al., 2008). However, endochondral bone formation, induced by BMPs, occurring in skeletal muscle appears to involve different cell types including fibroblasts, which contribute to the intermediated chondrogenic phase, and myogenic cells, which contribute to the osteogenic phase (Li et al., 2005). An analogous paradigm of distinct chondrogenic and myogenic cell populations residing in the same skeletal structures is observed in embryonic limb-bud development (Sasse et al., 1984; Swalla and Solursh, 1986; Kosher and Rodgers, 1987; Stringa et al., 1997). That is, a population of chondrogenic cells isolated from the limb-bud can be induced to differentiate into chondrocytes, but not to the myogenic lineage *in vitro*. Conversely, a population of myogenic cells isolated from the limb-bud can differentiate into myotubes (immature muscle fibers), but not chondrocytes (Swalla and Solursh, 1986). These results have been verified in clonal studies of these distinct

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populations, and mixed clones of definitive chondrogenic and myogenic cells have not been observed (Dienstman et al., 1974). Hence, it is reasonable to postulate that distinct populations of chondrogenic and myogenic progenitors are maintained throughout development and could be isolated from the mature limb.

Based on one of our previous studies (Li et al., 2005) and the latter studies performed on the embryonic limb-bud cells, we hypothesized that non-myogenic cells residing in the fascia of skeletal muscle may contain a population of cells with chondrogenic potential. By definition, the fascia of skeletal muscle represents fibrous connective tissue that comprises the outermost sheath of the skeletal muscle. In the present study, fascia-derived cells (FDCs) were isolated from the fascia of a Fischer 344 rat gluteus maximus muscle and investigated for their chondrogenic potential. FDCs are a heterogeneous population of cells including fibroblasts, endothelial cells from the vasculature, and various putative progenitor populations. In order to characterize the chondrogenic populations, FDCs were sorted into three subpopulations by FACS using two cell surface markers (CD29 and CD146). CD29 has been reported to be a cell surface marker of both mesenchymal stromal cells and chondrogenic progenitor cells (Wang et al., 2004; Wulf et al., 2004; Gimeno et al., 2005; Crisan et al., 2008; He et al., 2010); therefore, $CD29^+$ FDCs were expected to represent the cell fraction with the greatest chondrogenic potential. Furthermore, multiple studies have shown that there exist multipotent vasculature-related cells residing in skeletal muscle (Dellavalle et al., 2007; Zheng et al., 2007; Crisan et al., 2008). CD146 has been reported to be a marker of vasculature-related cells, endothelial cells, smooth muscle cells and pericytes (Bardin et al., 2001; Li et al., 2003; Middleton et al., 2005; Duda et al., 2006; Erdbruegger et al., 2006; Figarella-Branger et al., 2006). The expression of CD146 was utilized in the current study to determine if it could be correlated with the chondrogenic potential of the isolated cells. This sorting process yields four experimental groups: unsorted FDCs, $CD29^+CD146^-$ FDCs, $CD29^+CD146^+$ FDCs, and $CD29^-CD146^-$ FDCs ($CD29^-CD146^+$ FDCs were not detected). The *in vitro* chondrogenic potential of each population following treatment with BMP4 and TGF-β3 was investigated.

Skeletal muscle perimysium and endomysium are sheaths of connective tissue that segregate skeletal muscle fascicles and fibers, respectively, and have a similar histology, structure, and function to fascia, only at a different scale of muscle structure (Kragh et al., 2005; Kurose et al., 2006). It was further hypothesized that non-myogenic cells within skeletal muscle, likely associated with endomysium and perimysium, may possess chondrogenic potential. However, there is no known physical method to isolate these tissues from skeletal muscle given its superstructural complexity. Therefore, the presence of chondrogenic cells in skeletal muscle of a Fischer 344 rat gluteus maximus muscle was also investigated by isolating a heterogeneous population of muscle-derived cells (MDCs), which then were examined for the presence of cells with chondrogenic potential.

Finally, human FDCs were isolated from a gluteus maximus muscle fascia biopsy. It was hypothesized that, like rat muscle fascia, non-myogenic progenitors with chondrogenic potential exist within human skeletal muscle fascia. Given the limited effectiveness of current cartilage repair modalities, this study could lead to the identification of chondrogenic progenitor cells that could be harvested from the skeletal muscle fascia and used for cartilage regeneration.

Results

Staining of fascia tissue of gluteus maximus muscle and characterization of FDCs

H&E staining showed that fascia tissue is composed of fibrous and highly cellular connective tissue enveloping the skeletal muscle. Immunohistochemical staining of fascia tissue revealed that all fascia cells were vimentin (fibroblast marker) positive, a smaller fraction was CD29 positive or CD146 positive (Figure 1A). Freshly isolated FDCs were immunostained for a hematopoietic cell marker (CD45), endothelial cell markers (CD34, CD31, CD144, vWF, Flk-1 and CD146) and mesenchymal stromal cell markers (CD29, CD59, and CD90) and then analyzed by flow cytometry. All FDCs were negative for the hematopoietic cell marker CD45. Very few FDCs (<0.1%) expressed endothelial cell markers (CD34, CD144, vWF, Flk-1), although some CD31 (1.9%) and CD146 (1%) expression was observed. A large fraction of FDCs expressed the mesenchymal stromal cell makers CD59 (64.5%), CD29 (14.3%), and CD90 (5.45%) (Figure 1B).

When cultured in a monolayer in proliferation medium (PM), FDCs acquired a fibroblast-like appearance. In fact, when stained for desmin (myogenic cell marker) and vimentin, FDCs showed minimal positive staining for desmin yet were uniformly positive for vimentin (Figure 1C). A lack of myotube formation was observed in FDCs when these cells were cultured in myogenic differentiation medium (low serum culture medium), supporting the low myogenic potential of these cells. A lack of MyoD expression also supports the absence of myogenic cells in the FDCs (Figure 1C).

Characterization of FACS-sorted FDCs

FDCs were sorted by FACS immediately following the cells' dissociation. Once hematopoietic cells ($CD45^+$) were excluded, viable cells were gated and further sorted into the following subgroups: (i) $CD29^+CD146^-$ FDCs (39.2% of the total cell population); (ii) $CD29^+CD146^+$ FDCs (1.5% of the total population); (iii) $CD29^-CD146^-$ FDCs (21.4% of the total cell population). Note that there were no $CD29^-CD146^+$ FDCs detected (Figure 2A–C). The viable, $CD45^-$ sorted cells in each subgroup which were recovered for every experiment included 1.18×10^5 $CD29^+CD146^-$ FDCs, 1.7×10^3 $CD29^+CD146^+$ FDCs and 2.75×10^4 $CD29^-CD146^-$ FDCs. Purities of these three sorted cell populations were 97.7%, 93.8% and 98.0%, respectively, as confirmed by flow cytometry analysis performed immediately after FACS.

Proliferation rate of FACS-sorted FDCs

A significantly higher proliferation rate was observed in $CD29^+CD146^-$ FDCs compared with both $CD29^+CD146^+$ FDCs and $CD29^-CD146^-$ FDCs when cultured in DMEM supplemented with 1% FBS and 500 ng/ml BMP4 ($P < 0.01$). Additionally, $CD29^-CD146^-$ FDCs had a significantly higher rate of proliferation than $CD29^+CD146^+$ FDCs ($P < 0.01$) (Figure 2D). When cultured in DMEM supplemented with 10% FBS with BMP4, $CD29^+CD146^-$ FDCs, again, had a significantly higher rate of proliferation than both $CD29^+CD146^+$ FDCs and $CD29^-CD146^-$ FDCs

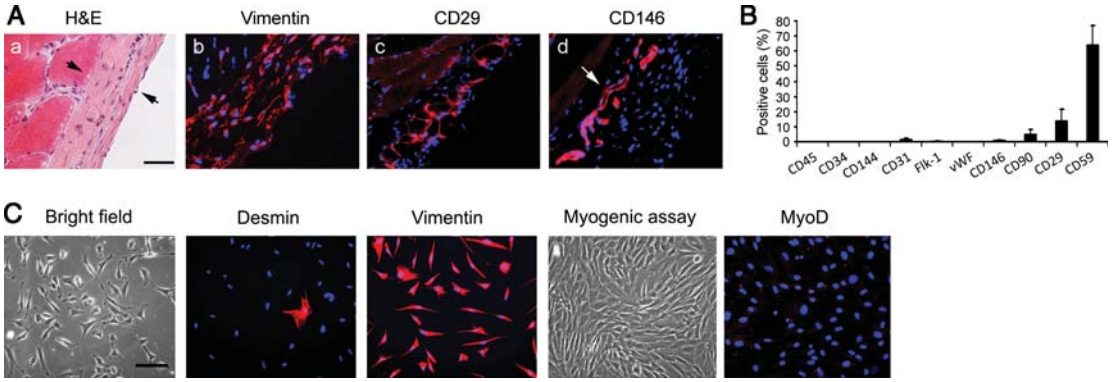

Figure 1 Characterization of fascia tissues and FDCs. (**A**) Staining of fascia. a, H&E staining; b–d, co-immunostained by vimentin, CD29, or CD146 antibody (red) and DAPI (blue). Fascia that surrounds skeletal muscle is indicated with black arrows. White arrow indicates tubular morphology of CD146 positive blood vessel. (**B**) Surface antigen profile of freshly isolated FDCs. (**C**) FDC morphology analysis. FDCs show a fibroblastic shape *in vitro* by the co-immunostaining with cell type marker desmin, vimentin, or MyoD antibody (red) and DAPI (blue). Scale bar, 50 μm.

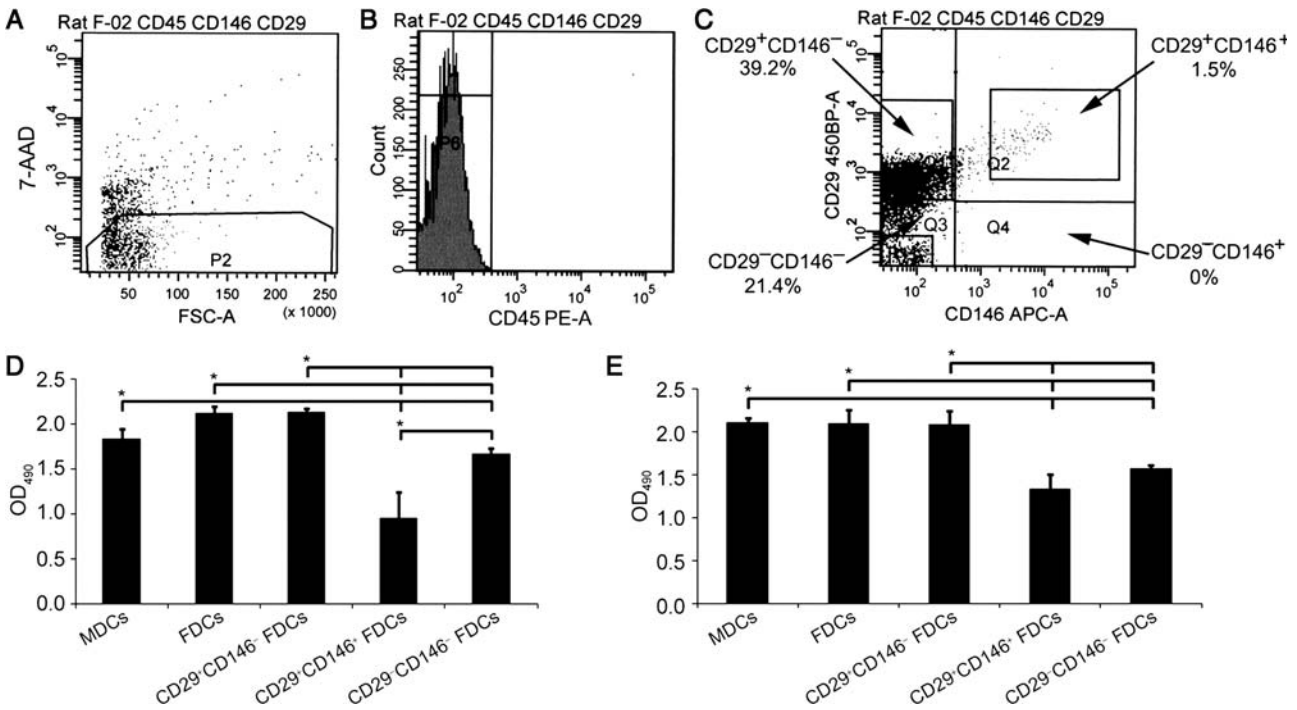

Figure 2 Characterization and proliferation potential of FACS-sorted FDCs. (**A**) Cell viability gates were set as indicated on the whole fascia cell suspension. (**B**) Negative selection of $CD45^+$ cells. (**C**) $CD29^+CD146^-$ FDCs, $CD29^+CD146^+$ FDCs and $CD29^-CD146^-$ FDCs were gated and sorted. (**D** and **E**) Proliferation rates of FDCs in DMEM with either 1% (**D**) or 10% FBS (**E**). *$P < 0.01$.

($P < 0.01$). However, in this case, no significant differences between $CD29^+CD146^+$ FDCs and $CD29^-CD146^-$ FDCs were observed (Figure 2E).

Chondrogenic and proliferation potential of FACS-sorted FDCs

The different FDC populations displayed different chondrogenic potentials as determined by extracellular matrix staining by both safranin O and Alcian blue. The order of decreasing chondrogenic potential was as follows: unsorted FDCs, $CD29^-CD146^-$ FDCs, $CD29^+CD146^-$ FDCs, and $CD29^+CD146^+$ FDCs (Figure 3A–C and Supplementary Figure S1). The round morphology typical of chondrocytes and positive extracellular matrix staining by both safranin O and Alcian blue were observed in three of the four groups (FDCs, $CD29^+CD146^-$ FDCs and $CD29^-CD146^-$ FDCs) at different time points (day 14, 21 and 28) but a lack of chondrogenesis was observed at any time point tested in the case of $CD29^+CD146^+$ FDCs (Figure 3B–E). These results were confirmed by immunostaining for collagen type II (Figure 3F). The four populations of cells also displayed different proliferation potentials in both PM and endothelial growth medium-2 (EGM2) after 2 weeks in culture (Figure 3G).

Characterization and chondrogenic potential of rat MDCs

MDCs expressed the mesenchymal stromal cell markers CD29 (10%), CD59 (12.9%), and CD90 (0.6%), whereas few cells expressed CD31 (1%) and CD146 (5%). MDCs were all negative for CD45 and very few (<0.1%) expressed endothelial cell markers (CD34, CD144, vWF, Flk-1) (Figure 4A). MDCs acquired

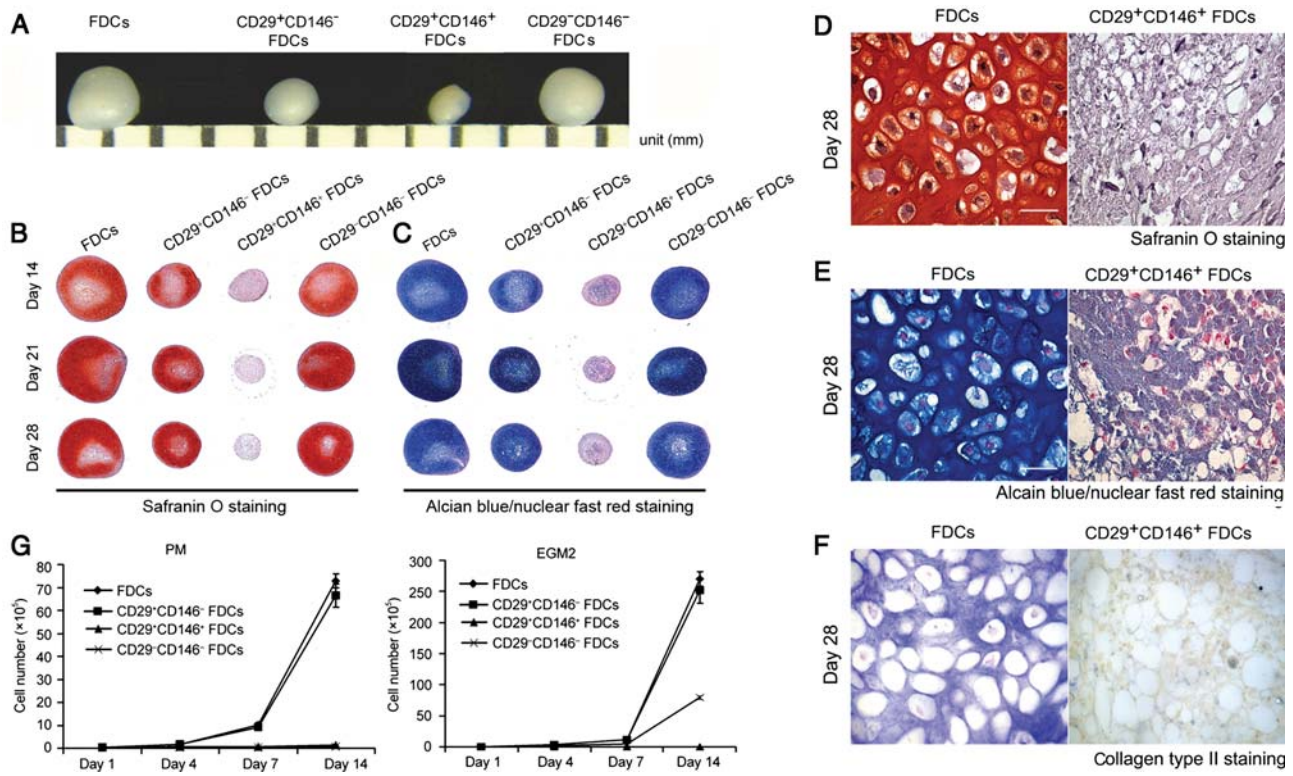

Figure 3 Chondrogenic and proliferation potential of FACS-sorted FDCs. (**A**) Macroscopic view of chondrogenic pellets. Safranin O (**B**) and Alcian blue/nuclear fast red (**C**) staining at different time points (days 14, 21 and 28). High-magnification safranin O (**D**) and Alcian blue (**E**) staining of FDCs and $CD29^{+}CD146^{+}$ FDCs. (**F**) Immunostaining for collagen type II of FDCs and $CD29^{+}CD146^{+}$ FDCs. (**G**) Four populations of cells displayed different growth potentials when cultured in either PM or EGM2. FDCs demonstrated the greatest proliferation potential while $CD29^{+}CD146^{+}$ FDCs displayed the lowest proliferation potential. Scale bar, 25 μm.

a spindle-shaped or fibroblast-like appearance in monolayer culture in PM, and showed 23% ± 2% positive staining for desmin and 100% positive staining for vimentin (Figure 4B). A myogenic differentiation assay demonstrated myotube formation in MDCs, as detected by MyoD immunostaining (Figure 4B). MDCs also demonstrated chondrogenic potential when cultured in chondrogenic medium supplemented with 500 ng/ml BMP4 and 10 ng/ml TGF-β3 at all measured time points (Figure 4C), indicated by positive extracellular matrix staining for both safranin O and Alcian blue as well as their round morphology typical of chondrocytes (Figure 4C–E).

Chondrogenic potential of mixed FDC and L6 myoblast pellets

A chondrogenic assay of pellets with varying ratios of FDCs and L6 myoblasts showed chondrogenic potential decreased with increasing ratio of L6 myoblasts to FDCs. Pellets composed entirely of FDCs showed the greatest chondrogenic potential, whereas pellets made entirely with L6 myoblasts demonstrated the least chondrogenic potential (Figure 5A). Representative images of safranin O, Alcian blue and collagen type II stained of FDC and L6 myoblast pellets indicate the high chondrogenic potential of FDCs, while L6 myoblasts display a limited chondrogenic potential (Figure 5B).

Chondrogenic potential of human FDCs

Human FDCs cultured in PM similarly acquired fibroblast-like spindle morphology. Immunostaining showed that very few human FDCs expressed desmin, whereas most of them expressed vimentin (Supplementary Figure S2A). Pellets of human FDCs demonstrated their chondrogenic differentiation potential (Supplementary Figure S2B–D), as indicated by positive safranin O, Alcian blue, and collagen type II staining.

Discussion

In the present study, we demonstrated that non-myogenic cells isolated from the skeletal muscle fascia possess chondrogenic potential following treatment with BMP4. These FDCs are distinct from myogenic progenitors isolated from skeletal muscle, previously shown by our group to undergo osteogenesis and chondrogenesis (Wright et al., 2002; Kuroda et al., 2006). These cells express most mesenchymal stromal cell markers and few endothelial cell markers. They are located in the epimysial fascia and within the muscle (likely the endomysium and perimysium). FDCs can be readily isolated from fascia and expanded *in vitro*, making them a readily accessible cell source for potential cell-based cartilage repair.

All FACS-sorted FDC fractions showed chondrogenic differentiation potential except the $CD29^{+}CD146^{+}$ FDCs, which constituted only 1.5% of the FDCs, which indicates that the majority of FDCs have chondrogenic potential following treatment with BMP4. Cell surface marker profiles were examined for potential correlation with the chondrogenic potential of their cell subpopulation. Indeed, we observed the following trend of chondrogenic differentiation potential (highest to lowest) for the experimental

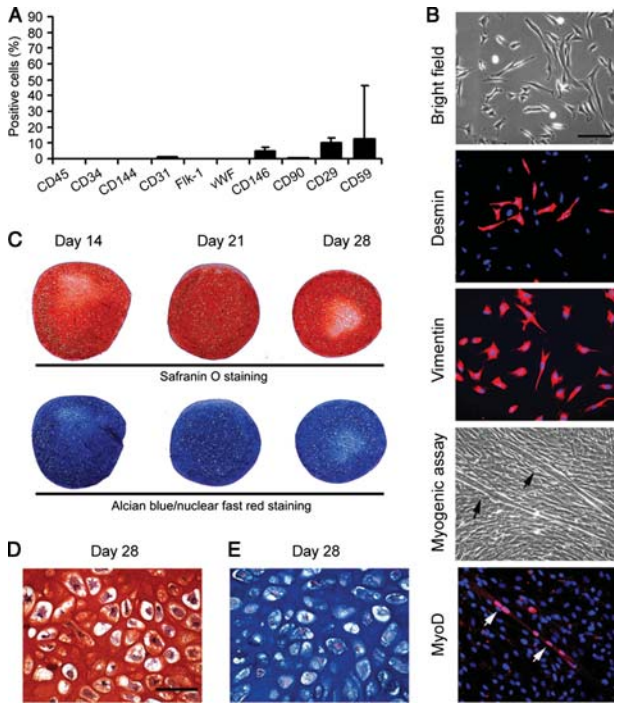

Figure 4 Characterization and chondrogenic potential of rat MDCs. (**A**) Surface marker profile of freshly isolated rat MDCs. (**B**) MDCs show a fibroblast shape by the co-immunostaining with cell type marker desmin, vimentin or MyoD (red) and DAPI (blue). Scale bar, 50 μm. (**C**) Safranin O (red) and Alcian blue staining (blue) of MDCs at day 14, 21 and 28. (**D** and **E**) High-magnification morphology of safranin O and Alcian blue staining of day 28 group. Scale bar, 5 μm.

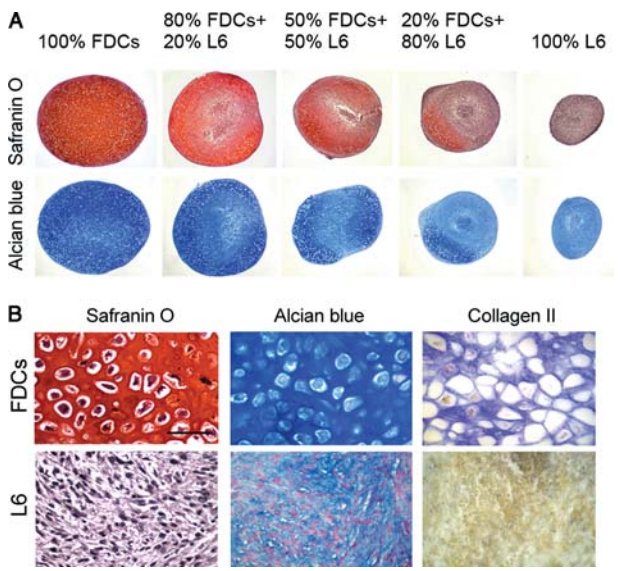

Figure 5 Chondrogenesis of mixed FDCs and rat L6 myoblasts. (**A**) Chondrogenic differentiation of pellets with varying ratios of FDCs and L6 myoblasts using safranin O and Alcian blue staining. (**B**) High-magnification morphology of safranin O, Alcian blue and collagen II staining of FDCs and L6 groups. Scale bar, 25 μm.

groups, including FDCs, $CD29^{-}CD146^{-}$ FDCs, $CD29^{+}CD146^{-}$ FDCs and $CD29^{+}CD146^{+}$ FDCs. The increased rate of chondrogenic differentiation of both $CD29^{-}CD146^{-}$ FDCs and $CD29^{+}CD146^{-}$ FDCs indicates that CD29 is not a major determinant cell marker of chondrogenic potential. Moreover, lack of chondrogenic differentiation in $CD29^{+}CD146^{+}$ FDCs, the only isolated cell population positive for CD146, implies that vasculature-related cells possess the least chondrogenic potential. However, this result did not exclude the possibility that these chondrogenic progenitor cells might originate from the vasculature because the cells may lose their chondrogenic potential after they fully differentiate into vasculature-related cells (CD146 positive). In addition, endothelial cell markers are not stable during *in vitro* expansion and one cannot exclude the presence of endothelial cells that are negative for endothelial cell markers (Zheng et al., 2007). Moreover, fascia tissue is also rich in blood vessels, which implies that these chondrogenic progenitor cells might reside in the 'stem cell niche' surrounding the blood vasculature. The blood cell walls may harbor a dormant reserve of chondrogenic progenitors that could be recruited when they receive a BMP4 stimulation signal.

Proliferation assays of the different FDC populations obtained after sorting did not correlate with chondrogenic potential; however, the unsorted FDCs endowed with the greatest chondrogenic potential also displayed the highest rate of *in vitro* proliferation in all media conditions tested (Figure 2D and E). This rapid rate of proliferation may be primarily attributed to the presence of $CD29^{+}CD146^{-}$ cells in the unsorted FDCs since a similar proliferation profile was observed between these two cell populations. The high chondrogenic potential of unsorted FDCs highlights the need of multiple cell types for optimal chondrogenic potential. It is likely that some of the cells within the unsorted fraction are responsible for chondrogenic differentiation, while others may be important for releasing signals involved with the maintenance and proliferation of that fraction of cells involved in chondrogenic differentiation. Results of the long-term proliferation of cells cultured in both PM and EGM2 also confirmed that unsorted FDCs have the strongest proliferation potential when compared with the other subpopulations of FDCs (Figure 3G). This characteristic demonstrated that the unsorted FDCs would be a good candidate cell source for cartilage repair clinically because large numbers of cells could be obtained in a relatively short period of time (3×10^7 cells in a 2-week period). Indeed, the need for multiple cell types for optimal progenitor or stem cell maintenance, proliferation, or differentiation has been observed in a variety of cell types, including embryonic stem cells, bone marrow stromal cells, skeletal muscle cells, etc. (Michalska, 2007; Carvalho et al., 2008; Neuhuber et al., 2008). Another aspect that could affect the chondrogenic potential of the cells is their time in culture or passage number. In the current study, we only used passage 5 from each of the populations and did not check the chondrogenic potentials of the later passages (>20). Based on the chondrogenic potential of the FDCs, we next attempted to demonstrate that FDC-like cells (cells derived from the endomysium and perimysium) in the skeletal muscle would also have similar chondrogenic potentials. Note that because the endomysium and perimysium are very hard to be physically separated from the skeletal muscle fibers because of their physical proximity to the fibers, all the MDCs were used. Therefore, we would expect

this MDC population represents a heterogeneous population of cells, which would include myogenic cells and connective tissue derived cells from endomysium and perimysium. MDCs did not show any significant difference in their cell marker profile via FACS analysis when compared with the FDCs. However, positive staining of the myogenic cell marker desmin (23% $\pm$ 2%) and myotube formation by the MDCs indicate that, as expected, a population of myogenic cells exists in MDCs, which was not found in the FDCs. In order to clarify whether endomysium and perimysium-derived cells play a role in MDC-mediated chondrogenesis, mixed pellets of FDCs and L6 myoblasts in different ratios (1:0, 4:1, 1:1, 1:4, 0:1 of FDCs to L6 cells) were tested for chondrogenic potential. Indeed our results demonstrated that the L6 myoblasts contributed negatively to the overall chondrogenic potential of the pellet, supporting our contention that connective tissue-derived cells (epimysium, perimysium and endomysium) are required for chondrogenesis. These results are consistent with our previous study (Li et al., 2005), in which a mouse myoblast cell line (C2C12 cells) demonstrated a low chondrogenic potential, whereas a fibroblast cell line (NIH/3T3 cells) was found to be highly chondrogenic.

Human fascia-derived cells showed similar characteristics to rat fascia cells in terms of cell surface markers and chondrogenic potential. Cells isolated from various human tissues have demonstrated chondrogenic potential, such as bone marrow (Mackay et al., 1998), adipose tissue (Estes et al., 2006), synovium (Sakaguchi et al., 2005), umbilical cord blood (Kogler et al., 2004), and human skeletal muscle (Mastrogiacomo et al., 2005); however, the chondrogenic potential of cells isolated from skeletal muscle remains controversial. One study reported that cells isolated from skeletal muscle have minimal chondrogenic potential when compared with the bone marrow, synovium, periosteum and adipose tissue (Sakaguchi et al., 2005). On the other hand, other studies demonstrated that skeletal muscle-derived cells (SMDCs) resemble bone marrow stromal cells and had the potential to form cartilage rudiment *in vitro* (Mastrogiacomo et al., 2005; Zheng et al., 2007). The discrepancies in these studies may be related to the procedures followed for cell isolation. Sakaguchi et al. (2005) used, collagenase D to digest the muscle, whereas trypsin was used in the latter study. It is possible that the majority of muscle-derived cells in the former study (Sakaguchi et al., 2005) are myogenic cells, while SMDCs are highly fibrogenic in nature and hence had a high chondrogenic potential (Mastrogiacomo et al., 2005). As expected, cells used by Mastrogiacomo et al. (2005) were reported to be more fibrogenic and less myogenic based on their surface marker expression (high vimentin and low desmin). Therefore, their study is consistent with our observations that human skeletal muscle fascia-derived cells, which are more fibrogenic (high expression of vimentin) and less myogenic (low expression of desmin), contain chondrogenic progenitor cells.

The *in vivo* disappearance of implanted cells that possess chondrogenic potential over a period of 6 months is a challenging aspect for applying the cells in articular cartilage repair. It appears that the majority of the implanted cells differentiate into hypertrophic chondrocytes and then eventually become apoptotic or calcified (Pelttari et al., 2006). It has been reported that human mesenchymal stromal cells (MSCs), the gold standard for *in vitro* chondrogenesis, can get through the hypertrophic chondrocyte stage when they are cultured *in vitro* with BMP2 or BMP4. The chondrogenic differentiation process of human MSCs *in vitro* is different from the natural development of articular cartilage chondrocyte differentiation in the joint, which acquires an intrinsic and stable chondrocytic stage without hypertrophy and becoming resistant to vascular invasion and calcification (Lian et al., 1993; Gerstenfeld and Shapiro, 1996; Steinert et al., 2009); however, the mechanism behind the chondrogenic differentiation cellular arrest process is still not fully understood. In the present study, the chondrogenesis of FDC pellets stimulated with BMP4 showed similar staining results for proteoglycans and glycosaminoglycans (both markers of hyaline cartilage) and the morphology of the chondrocytes was also consistent at three different time points (day 14, 21 and 28). No obvious changes, with regard to the sizes of chondrocytes lacuna, were observed when the early and later time points were compared; however, it is possible that there might be a size change associated with the chondrocyte lacuna as the cells become hypertrophic at longer time points (over 28 days).

To address the concern that the implanted cells possessed chondrogenic potential for cartilage repair diminish over time *in vivo*, we have performed earlier studies where the cells were retrovirally transduced with both BMP4 and sFlt1 (a VEGF antagonist) genes and were tested for their chondrogenic potential, both *in vitro* and *in vivo*, and compared with cells transduced with BMP4 only. The results showed that sFlt1 gene therapy improved the BMP4-induced chondrogenic gene expression of the cells *in vitro* and improved the persistence of articular cartilage repair in both a cartilage defect model (Kubo et al., 2009) and an osteoarthritis model (Matsumoto et al., 2009). The posited mechanism for sFlt1's action is that it prevents chondrocyte hypertrophy and apoptosis by blocking the intrinsic VEGF catabolic pathway *in vitro* and extrinsic VEGF-induced vascular invasion *in vivo*. Overall, the more we understand about the mechanisms of *in vivo* chondrogenesis, the more likely we will be able to create protocols to retain the transplanted cells in the chondrocytic stage and keep them from differentiating into hypertrophic chondrocytes.

The two main differences between the FDCs and human MSCs appear to be related to their dose-effect variability to BMP4. High dose of BMP4 (500 ng/ml) is needed to induce FDCs to differentiate into chondrocytes compared with the relatively lower dose of BMP4 (25 ng/ml) required to initiate the differentiation of MSCs (Steinert et al., 2009). Furthermore, initiating chondrogenesis in human MSCs does not even require BMP4 stimulation (Li et al., 2010). In addition, these two populations of cells displayed different ostogenic potentials, with the FDCs showing very poor osteogenic potential when cultured in osteogenic medium (data not shown), and the human MSCs being highly prone to differentiating into an osteogenic lineage (Jaiswal et al., 1997).

In summary, fascia, including the perimysium and endomysium in skeletal muscle, contain cells with high chondrogenic potential. These cells, which lack myogenic potential, express mesenchymal stromal cell markers and low levels of endothelial cell markers; therefore, we concluded that non-myogenic cells residing in the

fascia of skeletal muscle contain cells with chondrogenic potential. These results support the processes involved in endochondral bone formation within skeletal muscle following BMP stimulation, which appears to involve two cell types: fibroblastic cells that are directly involved in chondrogenesis and myogenic cells that are involved in the osteogenesis of the cells. Our results also suggest that skeletal muscle fascia represents a novel tissue source from which chondrogenic progenitor cells can be harvested for articular cartilage repair.

Materials and methods

Histological and immunohistochemical staining of skeletal muscle

Skeletal muscle was harvested from the gluteus maximus of Fischer 344 rats, embedded in CRYO-GEL (Cancer Diagnostics), rapidly frozen in pre-cooled 2-methylbutane and stored at −80°C. Muscle sections were fixed in 10% formalin at room temperature (RT) for 15 min, and then subject to H&E staining or immunostaining for vimentin, CD29 or CD146. For vimentin staining, sections were incubated at RT for 45 min with a Cy3-conjugated vimentin antibody (1:200 in PBS; Sigma). For CD29 and CD146 staining, sections were immersed in biotinylated hamster anti-rat CD29 (1:200 in 5% horse serum (HS); BD Pharmingen) and biotinylated goat anti-rat CD146 (1:200 in 5% HS; R&D System) for 2 h at RT, followed by streptavidin-Cy3 (1:500 in PBS; Sigma) for 45 min at RT. Sections were then incubated for 10 min in 4′,6-diamidino-2-phenylindole (DAPI) (1:1000 in PBS; Sigma), and examined under a fluorescent microscope.

Isolation and flow cytometry analysis of FDCs and MDCs

FDCs were isolated from the fascia of the left gluteus maximus of Fisher 344 rats, which was carefully detached from the muscle using surgical scissors. Both FDCs and MDCs were enzymatically isolated from their extracellular matrix using a method previously described (Qu-Petersen et al., 2002). They were resuspended in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin, triturated and passed through a 40-μm filter to obtain a single-cell suspension. FDCs and MDCs were divided into ten aliquots (1×10^5 cells) and resuspended in mouse serum (1:10 in PBS; Sigma) in 12 mm × 75 mm polystyrene flow tubes for 10 min on ice. Primary antibodies for the surface markers of interest (1:200) were added to the tubes. Each tube was treated with one primary antibody. These antibodies included fluorescein isothiocyanate (FITC)-conjugated mouse anti-CD34 (Santa Cruz Biotechnology), phycoerythrin (PE)-conjugated mouse anti-rat CD45 and CD31, and FITC-conjugated mouse anti-rat CD59 and CD90 (all from BD Pharmingen) for 30 min. Biotin-conjugated hamster anti-rat CD29, biotinylated goat anti-rat CD146 and biotinylated rabbit anti-CD144 (VE-cadherin; Alexis Biochemicals) were added to tube for 30 min and followed by streptavidin-APC (BD Pharmingen) on ice for another 30 min. Purified rabbit anti-Flk-1 (Santa Cruz Biotechnology) and rabbit anti-vWF (Chemicon) were then added to tubes for 30 min followed by FITC-conjugated goat anti-rabbit IgG (Vector Labs) and placed on ice for another 30 min. To exclude dead cells, 7-amino-actinomycin D (BD Pharmingen) was added to each tube. Live cells were analyzed using a FACS Calibur flow cytometer with CellQuest software (Becton Dickinson). The control samples obtained for each individual cell population received equivalent amounts of isotype antibody and were used to set the dot-plot intercepts for analysis.

Cell sorting of FDCs and cell culture

For cell sorting, FDCs were suspended in DMEM supplemented with 10% FBS, incubated with Pacific Blue anti-mouse/rat CD29 (Biolegend) and PE-conjugated mouse anti-rat CD45 and biotinylated anti-rat CD146 on ice for 30 min. Cells were washed with PBS supplemented with 2% FBS and incubated in streptavidin-APC (1:300) on ice for 30 min. Isotype control antibodies used were Pacific Blue Armenian hamster IgG (Biolegend), PE-conjugated mouse IgG1 (BD Pharmingen), and streptavidin-APC. 7-amino-actinomycin D was added to each tube for dead cell exclusion. Cell sorting was performed on a FACS multiple laser fluorescence cell sorter (Becton Dickinson) that was equipped with 488, 633 and 407 nm excitation wavelengths. Sorted cells were re-analyzed in all experiments. All cells were grown in 24-well plate in PM (DMEM supplemented with 10% FBS, 10% HS, 0.5% chick embryo extract and 1% penicillin/streptomycin) and were incubated at 37°C in humidified air with 5% CO_2.

Immunocytochemical staining and myogenic assay of FDCs and MDCs

FDCs and MDCs were cultured in PM in 6-well plates and fixed in cold methanol (−20°C) for 20 min. The cells were washed in PBS and blocked with 5% HS for 30 min. For desmin staining, a desmin antibody (1:200 in 5% HS; Sigma) was added to the cells for 2 h at RT. Following a PBS wash (three times for 5 min), anti-mouse IgG with a Cy3 conjugate (1:200; Sigma) was then added to the cells for 1 h at RT. For vimentin staining, cells were incubated for 45 min with Cy3-congugated vimentin antibody (1:200 in PBS) at RT, then washed in PBS and incubated for 10 min with DAPI solution (1:1000 in PBS). For the myogenic potential assay, FDCs or MDCs were cultured in PM until confluent and switched to a myogenic differentiation medium (DMEM with 2% HS) for 3 days. MyoD immunostaining was performed according to the above protocol using anti-MyoD (1:200 in 5% HS; BD Pharmingen) as primary antibody for 2 h at RT, followed by a PBS wash and addition of a biotinylated goat anti-mouse IgG (1:200 in PBS; Vector) for 1 h at RT. Finally, streptavidin-Cy3 (1:500 in PBS) was added to the cells for 45 min, as well as DAPI (1:1000 in PBS) solution for 10 min at RT. Cells were washed in PBS and examined under a DM IRB inverted fluorescent microscope (Leica), digitally recorded using a CCD camera (Qimaging Retiga), and analyzed using image analysis software (Eclipse).

Proliferation assay of sorted FDCs

Proliferation rates were measured for unsorted FDCs, $CD29^+CD146^-$ FDCs, $CD29^+CD146^+$ FDCs, and $CD29^-CD146^-$ FDCs using a colorimetric absorbance assay. Briefly, to determine short-term proliferation potential, cells were plated in 96-well plates (6000 cells/well) and cultured in DMEM supplemented with two different FBS concentrations (1% and 10%, the lower concentration will increase sensitivity of proliferation potential assay) and 500 ng/ml BMP4 (R&D Systems). After 2 days, 20 μl of CellTiter 96 aqueous one reagent (Promega) was added to

each well. The plate was incubated in 5% CO_2 at 37°C for 2 h. A 96-well plate reader was used to measure the absorbance at 490 nm.

Four populations of cells (unsorted FDCs, $CD29^+CD146^-$ FDCs, $CD29^+CD146^+$ FDCs, and $CD29^-CD146^-$ FDCs) were cultured up to 14 days in either PM or EGM2 to test their proliferation potentials. Cells of each population were plated at 6.5×10^4 cells/flask in T-25 flasks and cultured. They were counted and passaged into T-25 flasks where they grew to 60% confluence.

Chondrogenic differentiation of different FDCs and MDCs populations in vitro

Using a method described previously (Johnstone et al., 1998), 2.5×10^5 cells of each cell group (passage 5) were centrifuged at 500 *g* in 15-ml polypropylene conical tubes and the resulting pellets were cultured for up to 28 days in chondrogenic medium (Cambrex) supplemented with 500 ng/ml BMP4 and 10 ng/ml TGF-β3 (R&D Systems). The medium was changed every 2–3 days. The pellets were harvested on days 14, 21 and 28, embedded in paraffin, sectioned, and stained with safranin O (Sigma) and Alcian blue/nuclear fast red (Sigma) to determine the presence of proteoglycans (PGs) and gylcosaminoglycans (GAGs) indicated by a red color, and a positive reaction to sulfated PGs indicated by a blue color, respectively.

Chondrogenic assay for mixed pellets of FDCs and rat L6 myoblasts in vitro

The rat L6 myoblast cell line (CRL 1458; ATCC) was grown in T-75 flask in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at sub-confluence to prevent differentiation or myotube formation. FDCs and rat L6 myoblast cells were mixed and centrifuged to make pellets with different ratios (1:0, 4:1, 1:1, 1:4, 0:1; FDCs to L6 cells) with each pellet having the same total number of cells (2.5×10^5). These mixed pellets were generated to mimic the possible presence of the MDC population with FDCs (chondrogenic progenitors) and to study the effect of myoblasts on the chondrogenic potential of FDCs. Five groups of pellets were cultured in chondrogenic medium supplemented with BMP4 and TGF-β3 for 21 days. Pellets were embedded in paraffin, sectioned, and stained with safranin O and Alcian blue.

Collagen type II staining

Pellets were deparaffinized and processed for collagen type II immunostaining according to the manufacturer's protocol of Vecstastain ABC-AP kit (Universal) AK-5200 (Vector). Pepsin solution (Thermo Scientific) was used for enzyme-induced epitope retrieval from paraffin embedded sections. Collagen type II antibody (1:100; Thermo Scientific) was used for immunostaining. The Vector blue substrate kit (Vector) was used to reveal positive signal.

Isolation and analysis of human FDCs

Human FDCs were isolated from a fresh gluteus maximus muscle fascia biopsy, from a recently deceased cadaver, using the same method used to isolate rat FDCs (protocol approved by IRB). Human fascia was carefully removed from the attached gluteus maximus muscle and minced into a coarse slurry. The fascia tissue was then enzymatically dissociated at 37°C in 0.2% collagenase-type XI (Sigma-Aldrich) for 1 h and then centrifuged at 3500 rpm for 5 min. The pelleted cells were next incubated in 2.4 units dispase/ml Hank's balanced salt solution (HBSS) (Gibco) for 45 min, and then incubated for 30 min in 0.1% trypsin-EDTA (Gibco) in HBSS. After enzymatic dissociation, the human fascia cells were centrifuged and resuspended in PM. FDCs were immunostained for desmin and vimentin and their chondrogenic potential was evaluated after BMP4 treatment.

Statistical analysis

Data are expressed as the mean ± SD, except where noted. Direct comparisons between two cell populations were made using an ANOVA analysis. Statistical significance was assigned if $P < 0.05$.

Supplementary material

Supplementary material is available at *Journal of Molecular Cell Biology* online.

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