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Reflections on lineage potential of skeletal muscle satellite cells: Do they sometimes go MAD?

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Abstract

Postnatal muscle growth and repair is supported by satellite cells - myogenic progenitors positioned between the myofiber basal lamina and plasma membrane. In adult muscles, satellite cells are quiescent but become activated and contribute differentiated progeny when myofiber repair is needed. The development of cells expressing osteogenic and adipogenic genes alongside myoblasts in myofiber cultures, raised the hypothesis that satellite cells possess mesenchymal plasticity. Clonal studies of myofiber-associated cells further suggested that satellite cell myogeneity and diversion into Mesenchymal Alternative Differentiation (MAD) occur in vitro by a stochastic mechanism. However, in vivo this potential may be executed only when myogenic signals are impaired and the muscle tissue is compromised. Such a mechanism may contribute to the increased adiposity of aging muscles. Alternatively, it is possible that mesenchymal interstitial cells (sometimes co-isolated with myofibers), rather than satellite cells, account for the nonmyogenic cells observed in myogenic cultures. Herein, we first elaborate on the myogenic potential of satellite cells. We then introduce definitions of adult stem-cell unipotency, multipotency and plasticity, and elaborate on recent studies that established the status of satellite cells as myogenic stem cells. Lastly, we highlight evidence in favor of satellite cell plasticity and emerging hurdles restraining this hypothesis.

Keywords

Mesenchymal stem cell; myoblast; adipocyte; myogenesis; adipogenesis; osteogenesis; multipotential

What is this review all about and what is the MAD path?

Ever since their discovery, skeletal muscle satellite cells, situated underneath the myofiber basal lamina, have been considered as myogenic precursors destined to give rise to myoblasts needed for myofiber growth and repair^{1–3}. Indeed, early studies of isolated myofibers unequivocally established the myogenic potential of satellite cells^{4–7}. The recent finding that satellite cells can self-renew, in addition to producing proliferating and differentiating myogenic progeny, further established their myogenic stem cell status⁸.

The question of satellite cell plasticity, nevertheless, is still under a cloud. Several studies with cultures emanating from individual myofibers demonstrated the appearance of fat-containing cells as well as the expression of genes or proteins that are associated with osteogenic and adipogenic pathways in addition to myogenic differentiation^{9–11}. Moreover, cells transiting through sequential steps of adipocyte differentiation (culminating with mature adipocytes) were detected alongside cells undergoing myogenic differentiation in myofiber cultures¹². The latter study established that emergence of the alternate adipogenic cell type reflects a true developmental process and not just elevated gene expression within myogenic cells. With the notion that satellite cells are the sole entities that can produce daughter cells in cultures of isolated myofiber, the four aforementioned studies raised the possibility that satellite cells are multipotent stem cells, able to give rise to several mesenchymal lineages. It was further suggested that the different culture conditions that support the development of mesenchymal, nonmyogenic cells, are of physiological relevance, reflecting signaling cues that might impair the myogenic fate of satellite cells in vivo^{10–12}.

Importantly, our clonal studies of myofiber-associated cells suggested that diversion from myogenesis to mesenchymal alternative differentiation occurs within the satellite cells themselves and not within their progeny (i.e., before satellite cells produce daughter cells). We termed the proposed capacity of satellite cells to enter a **Mesenchymal Alternative Differentiation** program with the acronym MAD¹². The acronym MAD was originally used with a somewhat different context in a review suggesting that aging cells (myoblasts included) display enhanced expression of adipogenic genes - this process was termed Mesenchymal Adipogenic Differentiation¹³. However, before categorizing satellite cells or their immediate progeny as multipotent, it is essential to consider the possibility that interstitial cells, often co-isolated with myogenic cells, may account for the nonmyogenic cell types that developed in myogenic cultures. Additionally, cells from the capillary networks that are in close contact with myofibers (i.e., vessel wall and circulatory cells) and even resident macrophages or intramuscular mesenchymal stem cells can all be contributory cells^{12,14,15}.

The findings that myogenic cell lines can also adopt nonmyogenic mesenchymal fate were used as further support for satellite cell plasticity (discussed in ref 12 and later in this review). Nevertheless, these immortal cells underwent many changes over their long-term propagation, and thus could not be considered equivalent to satellite cells or primary myoblasts. It is crucial therefore to recognize the differences between myogenic cell lines and bona fide satellite cells, and to establish claims of plasticity solely on direct evidence from experiments with satellite cells. Moreover, in order to claim multipotency or plasticity of satellite cells it is essential to document the complete process of differentiation toward nonmyogenic cell types, as well as the gaining of alternate functionality of these cells. Overexpression of selected genes that are typically associated with the differentiation of nonmyogenic lineages does not necessarily imply that cells switched from one lineage to another (e.g., a cell that expresses alkaline phosphatase is not necessarily a committed osteoblast).

In this review we first elaborate on the myogenic potential of satellite cells. We then introduce definitions of adult stem cell unipotency, multipotency and plasticity, and elaborate on recent studies establishing that satellite cells are myogenic stem cells as they can both self-renew and produce myogenic progenitors. This is followed with a description of existing evidence of satellite cell molecular heterogeneity and a discussion about the classically held view of the mesodermal (somitic) origin of satellite cells versus the hypothesis that satellite cells in the adult can be contributed by additional sources, including adult mesenchymal stem cells. Lastly, we highlight evidence in favor of satellite cell

plasticity and emerging hurdles restraining this hypothesis. This review does not aim to provide a lengthy description on the regulation of satellite cell myogenesis. Rather, we aim at emphasizing limitations of studies that suggest satellite cell multipotency or plasticity compared to the solid evidence for the myogenic potential of these muscle stem cells. We conclude with reflections on future experimental directions for testing the hypothesis of satellite cell plasticity.

Satellite cells support myofiber integrity in adult skeletal muscle

The skeletal muscle tissue is composed of myofibers, multinucleated syncytia, that express highly-specialized contractile proteins. Myofibers are established primarily during embryogenesis by fusion of myoblasts with each other. Skeletal muscles continue to grow postnatally until the mature state is reached. In response to activities that induce subtle injuries such as exercise and work overload, or as a result of major trauma, muscles mount a highly orchestrated regenerative response to rapidly restore their cytoarchitecture^{16,17}. Myofiber nuclei are typically postmitotic and cannot contribute to muscle restoration. The remarkable repair capacity of myofibers is supported by cells whose unique location underneath the myofiber basal lamina led to terming them “satellite cells”^{1,3,18}. In the adult, satellite cells are typically quiescent, but they enter the cell cycle and produce myoblasts in response to stimuli generated by muscle damage. Subtle damage elicits a minimal proliferative response whereas major trauma stimulates satellite cells to generate a large pool of proliferating progeny that fuses to form new myofibers¹⁴. Functional satellite cells are present even in muscles of senile animals, but their number is far reduced compared with the postnatal growth phase^{19–21} (earlier studies are reviewed in ref 16). While the extracellular environment does not support efficient performance of satellite cells in old age, satellite cells themselves (at least some of them) maintain myogenic stem cell potential to very old age^{21–24}.

The myogenic program of satellite cells at the molecular level

Myogenesis of satellite cells is regulated by a family of muscle specific transcription factors (i.e., muscle regulatory factors or MRFs) that are expressed in a temporarily ordered manner and initiate a cascade of events that culminates in myoblast differentiation and fusion³. This family of transcription factors includes MyoD, Myf5, myogenin and MRF4²⁵. MRFs received different names when identified at several laboratories and in various species. For example, MyoD and myogenin were also termed Myf-3 and Myf-1, respectively, and MRF4 was also termed Myf-6 or Herculin^{26, 27}.

Quiescent satellite cells express the paired-homeobox transcription factor Pax7^{8,21,28} and are thought not to express any of the MRFs. Nevertheless, Myf5-driven reporter expression suggests that Myf5 gene is active in satellite cells²⁹. In accordance, our recent studies documented a relative high-level expression of endogenous Myf5 transcripts in quiescent satellite cells³⁰. MyoD expression is upregulated upon satellite cell activation and is maintained during subsequent proliferation and differentiation phases⁷. Differentiation commitment of myoblasts is marked by the onset of myogenin expression concomitantly with a decline in Pax7 and Myf5 expression, cell cycle withdrawal and subsequent fusion of myoblasts into multinucleated myotubes^{3,18,19}. The status of MRF4 during myogenesis of satellite cells is less clear as evidence exist for its expression before, after and together with myogenin. Members of the myocyte enhancer factor 2 (MEF2) transcription factor family are also involved in the regulation of myogenesis^{31,32}. After differentiation commitment, MRFs function in concert with MEF2s to support the expression of muscle specific structural proteins. Differentiating progeny of satellite cells exhibit enhanced expression of MEF2A in correlation with the onset of myogenin expression^{33–35}.

Myoblasts entering differentiation commitment can still proliferate and their terminal differentiation requires permanent withdrawal from the cell cycle^{36,37}. This terminal myogenic differentiation is controlled by cell-cycle regulators such as cyclin-dependent kinase, cyclinD3, and cyclin-dependent kinase inhibitors p21 and pRb. The latter is involved in the regulation of both cell cycle withdrawal and expression of genes encoding for structural proteins^{38,39}. MyoD was suggested to govern the induction of the abovementioned cell-cycle regulators^{40,41}, implying that MyoD is kept in a transcriptionally inactive form until the appropriate signals for inducing differentiation are conveyed^{42–46}.

Satellite cells (or at least some of them) fulfill the definition of stem cells

A bona fide stem cell is generally defined as an undifferentiated cell that can undergo asymmetric division to give rise to (i) a new stem cell (process termed self-renewal); and (ii) a daughter precursor cell, which is committed to proliferate before differentiation (referred to, among other terms, as a transit amplifying cell, proliferating precursor or progenitor cell)^{47–49}. This dual capacity to self-renew and to contribute differentiated progeny enables adult stem cells to support ongoing tissue homeostasis and contribute to tissue regeneration following trauma.

An adult stem cell is classified as unipotent when it produces only one cell type, typically of importance for the homeostasis of the tissue it resides in. An adult stem cell is classified as multipotent when it can produce more than one cell type, and the different progeny are of some relationships to each other^{15,50}. For example, hematopoietic multipotent stem cells produce several different cell types, all of relevance to the hematopoietic lineage. The term multipotency is often used in association with stem cell plasticity. While a widely accepted definition of stem cell plasticity has yet to be established, plasticity in general refers to the ability of adult stem cells to adopt the expression profiles and functions of cells unique to tissues and lineages other than that of the stem cells' host tissue⁵¹. This contention on plasticity has to be considered in light of two possible avenues: (1) a more liberal definition of plasticity refers to individual stem cells, which can give rise to cell types that do not share obvious lineal relationships or embryonic ancestor (e.g., development of neuronal cells from non-neuronal or non-ectodermal in origin stem cells); and (2) a more conservative/stringent definition refers to the capacity of a stem cell to give rise to more than one cell type, not necessarily with obvious lineal relationships but within the constraints of same germ layer (e.g., mesenchymal stem cells may give rise to myogenic, adipogenic and osteogenic progenitors). Further confusion presents itself when the term plasticity is used in connection with the term transdifferentiation. Plasticity and transdifferentiation are not interchangeable terms since the latter describes a differentiated cell that assumes a phenotype and function of another differentiated cell (e.g., smooth muscle cell lines spontaneously adopting a skeletal muscle phenotype in culture⁵²)^{50,53,54}. Another process termed dedifferentiation was shown to serve as an intermediate stage for producing new cell phenotypes, especially during whole organ regeneration in amphibians but dedifferentiation is not a typical process associated with regeneration in mammals^{55,56}.

The hypothesis that satellite cells undergo self-renewal was proposed more than 30 years ago⁵⁷ but a direct approach to investigate this topic has been introduced only recently⁸. In the latter study, single myofibers were isolated from mice in which one of the Myf5 alleles was modified to drive expression of nuclear lacZ (i.e., Myf5^{nlacZ/+} mice). This genetic manipulation allows tracing nuclei of satellite cells in muscle sections and isolated myofibers based on β -galactosidase expression²⁹, and further enables the detection of donor-derived Myf5^{nlacZ/+} satellite cells after transplantation to host muscles. Importantly, in the study of Collins and colleague⁸, the host animals were double mutant nude/mdx mice that were exposed to γ -irradiation a few days before transplantation. These features provided an

excellent environment for satellite cell transplantation for the following reasons: (i) mdx mice suffer from muscular dystrophy due to a mutation in the gene encoding dystrophin (therefore lacking this protein) and their myofibers undergo rounds of degeneration and regeneration which encourage participation of transplanted satellite cells in the continuous myofiber repair; (ii) the immune system of nude mice is compromised, ensuring tolerance to transplanted cells⁵⁸; and (iii) γ -irradiation results in ablation of the majority of endogenous satellite cells, increasing the frequency at which donor satellite cells participate in myofiber repair⁵⁹. Individual myofibers were transplanted in 3–4-week old host mice, as at this age the major bout of myofiber damage-repair occurred. Collins and colleagues⁸ also promoted muscle damage and subsequent regeneration by administering myotoxin to host muscles. In most cases, the transplantation of an individual myofiber (i.e., containing several satellite cells) into host hindlimb muscles resulted in vast amplification of the donor satellite cells. The amplification was manifested in a large number of newly-formed or repaired myofibers. New myofibers were distinguished by several cytological means (such as re-expression of dystrophin). Moreover, satellite cells of donor origin were identified in the native satellite-cell position, underneath the myofiber basal lamina. These satellite cells were functional and responded to injury by contributing myoblasts to a second round of muscle repair, providing the first direct demonstration that satellite cells self-renew in vivo.

The number of satellite cells varies widely between myofibers from the same muscle and between myofibers from different muscles^{8,21,60}. Satellite cell numbers are typically determined on fixed myofibers, based on expression of molecular markers that distinguish between the satellite cells and their parent myofiber. Notably, in the Collins et al. study⁸, living myofibers were transplanted and the number of satellite cells associated with each donor myofiber was not known. Thus, it is unclear if all satellite cells were equally able to self-renew and give rise to progeny for myofiber repair. To determine if all or some satellite cells possess a self-renewal potential, the experimental design of Collins and colleagues⁸ would require transplanting individual satellite cells. Additionally, the latter in-vivo model also does not permit investigating the mechanisms that govern satellite cell renewal.

Our current studies with a transgenic mouse model in which all satellite cells, but not their proliferating and differentiating progeny, express green fluorescent protein (GFP) enabled us to detect self-renewal of satellite cells in myofiber cultures and in clones of individual satellite cells³⁰. In these culture models, we detected re-expression of GFP only in quiescent cells expressing Pax7 (but not MyoD). Such GFP⁺ cells re-appeared only in cultures comprising dense networks of myotubes, always located in close association with myotubes. These data suggest a role for the myofiber in governing acquisition of the satellite cell phenotype³⁰. Using this model, we now study the molecular pathways that control the process of satellite cell-self renewal.

Importantly, our studies suggest that satellite cell renewal does not occur at the level of the satellite cell but rather in its progeny. An early study suggested that satellite cell renewal might occur within the initial proliferative rounds of satellite cells or their progeny; this was based on the detection of Pax7⁺/MyoD⁻ cells during early culture days⁶¹. Our observations that proliferation may be a prerequisite for satellite cell renewal argue against the notion that the satellite cell is a bona fide stem cell that divides asymmetrically to one committed daughter cell and one satellite stem cell. Nevertheless, it is possible that our cell culture model does not recapitulate signaling events that occur during muscle growth and routine repair, events that permit self-renewal of satellite cells per se. Our model may rather reflect events occurring during major muscle injury, when massive amounts of myogenic cells are required in order to form myofibers de novo. In this case, an initial amplification phase is required, and only subsequently, after myoblasts fuse into myofibers, other residual myoblasts may acquire satellite cell properties.

Molecular heterogeneity of satellite cells within and between muscles suggests that they do not encompass a homogeneous population

The original definition of satellite cells is based on their anatomical position¹. According to this definition, all cells located beneath the basal lamina of a myofiber are satellite cells regardless of their function or gene expression profile. Nevertheless, in situ analysis of satellite cells based on their expression of a set of characteristic molecular markers demonstrated that not all satellite cells uniformly express all markers³. Most satellite cells share a common molecular signature and express Pax7, Myf5-driven lacZ, M-cadherin and CD34 (CD34 is also expressed by other cell types in the muscle)^{29,62}. Co-expression analyses for these markers, however, typically identify residual populations that are positive for some but not all markers^{29,30}.

The observed molecular heterogeneity within the same muscle (and even the same myofiber) may point to different metabolic phases of the presumably quiescent satellite cells at the time of testing. However, intermuscular variations in satellite cell proliferative rates or in expression of differentiation-linked genes by their progeny may reflect inherent differences due to distinct embryonic origins or fiber type composition (i.e., ratio between the different fast- and slow-twitch myofibers) of the hosting muscles^{63–65} (reviewed in ref 3). To date, the unique high-level expression of Pax3-driven reporter⁶⁶ or of endogenous Pax3 gene³⁰ by satellite cells in diaphragm muscle versus marginal Pax3 expression in most hindlimb muscles presents the most convincing evidence for intermuscular heterogeneity of satellite cells. Pax3 and Pax7 constitute one of the four Pax gene subfamilies and both are expressed during embryonic muscle development^{67,68}. There is no clear explanation for this differential Pax3 expression, as it is not related to muscle ontogeny or fiber type composition. Moreover, when GFP⁺ satellite cells from donor Pax3^{GFP/+} mice were transplanted into host hindlimb muscle that usually does not contain Pax3-expressing satellite cells, donor-derived cells that were detected in the satellite cell position expressed GFP⁶⁹. These data raise the possibility that Pax3^{GFP/+} donor satellite cells have intrinsic characteristics that are retained even in a different cellular environment. The exact implication of this intramuscular heterogeneity in satellite cells and the question whether heterogeneity points to differences in satellite cell function remain unresolved at present time.

Are all satellite cells of mesodermal origin?

It is still unknown if all satellite cells of the adult muscle derive from a common ancestor and this subject has been under debate in recent years. The prevailing hypothesis is that satellite cells are derived from the somites, transitory mesoderm-derived structures formed in pairs on either side of the neural tube^{67,70–73}. As somites mature, they partition into the mesenchymal sclerotome (ventrally) and a dermomyotome (dorsally); the latter retains an epithelial nature. In amniotes, the four edges (also referred to as lips) of the dermomyotome are the main source of early myogenic cells that migrate to form the myotome (the first skeletal muscle fibers in the body) underneath the dermomyotome^{74–79}. The myotome gives rise to the epaxial muscles (deep back muscles) and to myogenic cells that migrate to the diaphragm and to the appendages (where they form limb musculature). The origin of satellite cells in head muscles is thought to be non-somitic; most head muscles do not derive from somites but from the pre-chordal mesoderm, and have a distinct genetic network that controls their formation⁸⁰.

The above studies on the embryonic origins of satellite cells were extended to 4–6-week old animals, and indeed support the somitic origin of satellite cells during postnatal growth. These studies, however, do not fully establish that all satellite cells in the adult are of

somitic origin. Cells from bone marrow, brain, adipose tissue, sinovia and muscle interstitium were suggested to possess a myogenic potential and in rare cases to even enter the satellite cell niche^{81–87}. There are various explanations for the development of myogenic cells from such nonmuscle sources, including: (i) existence of residual somite-derived myogenic cells in nonmuscle organs; (ii) transdifferentiation of cells from a respective tissue towards myogenesis; or (iii) existence of a multipotent stem cell residing in- or out-side the muscle tissue that can undertake a myogenic differentiation pathway. In most studies, the contribution of differentiated myoblasts from nonmyogenic origins is rare, and the basis for this phenomenon is poorly understood. Nevertheless, an ongoing debate regarding the origin of satellite cells in adult muscles^{3,18} was stimulated by the above findings, as well as by the further identification of interstitial cells that reside within the muscle tissue and can enter myogenesis, at least following injury⁸⁸.

Notably, cells of non-somitic origin shown to give rise to myogenic cells were also suggested to be multipotential. For example, mesoangioblasts that were isolated from dorsal aorta of mouse embryos were shown to be multipotent in nature, giving rise to functional myogenic cells as well as to cells of other lineages^{89–91}. The proposed mechanism of mesangioblast action is that upon transplantation they are lodged in muscle capillary beds, then the penetrate the muscle tissue, adopt a muscle fate, and contribute to its histogenesis. It is noteworthy that the dorsal aorta contains angioblasts from a somitic origin that contribute to the endothelial lining of vessels, as well as to the roof and walls of the aorta^{92, 93}. This raises the possibility that aorta cells that give rise to myogenic cells are, after all, of somitic origin (reviewed in ref 18).

In summary, the possibility of distinct developmental origins of satellite cells offers an attractive explanation for the observed molecular heterogeneity within the satellite cell compartment. Such distinct origins could potentially account for the apparent plasticity of satellite cells, based on the development of different mesenchymal cell lineages in myofiber cultures (discussed at greater details below). It is, however, unlikely that circulating nonmyogenic cells contribute functional satellite cells in the adult in view of the marginal, if any, detection of donor cells of hematopoietic origin in the satellite cell niche⁹⁴. Thus, even if such process does occur, it is an extremely rare event compared to the vast contribution of donor satellite cells to muscle repair and satellite cell renewal⁸.

Plasticity of mesenchymal stem cells and their lineage committed progeny in adult tissues

We discussed above the somitic origin of satellite cells, and since somites are derived from mesenchymal cells⁷⁶, it is conceivable that satellite cells may retain some mesenchymal plasticity similar to other mesenchymal-derived cells and mesenchymal stem cells. Below we elaborate in brief on the range of mesenchymal plasticity in adult tissues. A pioneering work by Friedenstein and colleagues demonstrated that cells from the bone marrow stroma could be grown ex-vivo and maintain their differentiation capacity in vivo upon re-implantation under the kidney capsule⁹⁵. This study led to the identification of mesenchymal stromal cells from the bone marrow that (i) support the formation of long-term hematopoietic activity; (ii) support hematopoietic stem cell renewal in culture; and (iii) have the potential to differentiate to chondro-osteogenic lineages. The bone marrow mesenchymal-derived cells adhere to plastic and differentiate under defined in vitro conditions into a spectrum of specialized mesenchymal tissues including bone, cartilage, muscle, marrow stroma, tendon, ligament, fat and a variety of other connective tissues^{96–99}. Only 0.001–0.0001% of nucleated cells in adult bone marrow are considered to be mesenchymal stem cells (MSCs). Mesenchymal-derived stem cells reside in the bone marrow and around blood vessels, and it

was suggested that they actually share identity with pericytes, smooth muscle like cells associated with the microvasculature^{100, 101}.

Pericytes are typically recruited from the local mesoderm by the endothelial network, and like MSCs can give rise to several mesenchymal cell types. Pericytes were shown to have osteogenic, chondrogenic and adipogenic potential^{102–104}. MSCs can also be present in muscle, fat, and synovium^{15,105,106}. Mature cells of mesenchymal origin transdifferentiate within constraints of mesenchymal cell lineages: chondrocytes and adipocytes switched their phenotype to that of osteoblasts^{107,108}. Fat cells were suggested to transdifferentiate into other cell types, including myoblasts^{82,109}. In the same vein, cells in myogenic cell lines can adopt other mesenchymal phenotypes when treated with various inducers (see next section).

Evidence on plasticity of the mesenchymal stem cells per se also accumulated from in vivo studies. Following transplantation into irradiated animals, MSCs engraft in the bone, cartilage, and lungs of mice¹¹⁰. Human MSCs that were transplanted in utero in sheep, contributed chondrocytes, adipocytes, differentiated myoblasts, cardiomyocytes, bone marrow stromal cells, and thymic stroma¹¹¹. Altogether, mesenchymal cells show lineage flexibility both as mature (lineage committed) and stem (pre-committed) cells. Adult MSCs are attractive candidates for stem-cell therapy due to their multipotency and their easy and safe collection from bone marrow. Nevertheless, they rarely differentiate into the skeletal muscle lineage and when such differentiation did occur, it was typically associated with a severe damage in the target muscle¹¹². The rarity of bone marrow cells that participated in muscle regeneration, even when muscles were injured in order to enhance regenerative processes, raises concerns for their physiological relevance for muscle regeneration^{3,112}. Another limitation of bone marrow/MS cell transplantation for stem-cell based muscle therapy is that for the most part, bone marrow-derived cells do not acquire a myogenic phenotype and do not upregulate expression of muscle specific genes, even when fusion with myofibers is well documented^{87,113–115}. To date, the only stem cells proven to contribute cells in physiologically relevant amounts upon transplantation are the skeletal muscle satellite cells⁸. Stem cells of other origins may still contribute nuclei for myofiber repair, as required during routine muscle utilization or during severe trauma. Understanding cues that elicit significant recruitment of MSCs or other bone marrow-derived cells to the muscle tissue awaits future studies.

Myogenic cell lines can be diverted from the myogenic differentiation pathway by lineage inducing agents

The idea of mesenchymal plasticity of myogenic cells was suggested in earlier studies with rodent myogenic cell lines. Cultures could be diverted from the myogenic path and generate cells of non-muscle mesodermal lineages such as osteoblasts and adipocytes. The diversion from the myogenic phenotype was induced by supplementing cultures with bone-morphogenesis proteins (BMPs) or adipogenic-inducing agents. For example, BMP-2 supplementation to the medium of C2C12 mouse myogenic cells was shown to result in the induction of the osteogenic molecular program based on upregulation of the master transcriptional regulators Runx2 and Osterix, parathyroid hormone response, and osteocalcin production^{116,117}. Moreover, BMP-2 converts the differentiation pathway of mouse C2C12 and rat L6 myoblasts into that of osteoblast lineage cells, by inhibiting MyoD and myogenin expression and preventing terminal myogenic differentiation^{116,118}.

In different examples, inhibition of myogenic differentiation and transition to adipogenic-like cells occurred via down-regulating the MyoD gene family in myogenic cells upon transfection with the adipogenic master regulators peroxisome proliferating activating receptor gamma2 (PPAR γ 2) and CCAAT-enhancer binding protein α (C/EBP α), or with the

dominant negative (DN) MKK3 in the presence of the chemical PPAR γ activator rosiglitazone^{119,120}. Moreover, supplementation of chemical or natural activators of PPAR γ to media of myogenic cell lines was sufficient to induce phenotypic and genotypic switch from myogenic to adipogenic-like cells. Specifically, cells accumulated triglycerides and genes involved in fatty acid uptake, storage, and metabolism were upregulated^{121–123}. C2C12 myoblasts also differentiated into the adipogenic lineage when the Wnt-signaling pathway was blocked¹²⁴.

On one hand, it could be argued that aforementioned studies point to some degree of plasticity within the mesenchymal lineage. This apparent plasticity could in fact reflect transdifferentiation, a process that occurs in other types of committed mesodermal cells¹²⁵. On the other hand, results from studies with cell lines are not necessarily fully applicable for assessing stem cell characteristics. In context of the myogenic program, characteristics of myogenic cell lines do not completely mirror the biology of satellite cells in terms of expression of master regulatory genes and with regards to their response to growth factors. For example, proliferating myoblasts of rat myogenic cell lines L6 and L8¹²⁶ express very little, if any, MyoD⁵². Additionally, these cell lines and the mouse myogenic cell line C2¹²⁷ express high level of platelet-derived growth factor (PDGF) receptors and their proliferation is regulated by PDGF ligands^{128–130}, whereas PDGF does not exert such mitogenic effects on myogenic cells in primary cultures of mouse and rat satellite cells. Only nonmyogenic cells, typically present in primary myogenic cultures, displayed mitogenic response to PDGF (Yablonka-Reuveni, unpublished results). Similarly, the growth factors IGF1 and IGF2 enhance both proliferation and differentiation of myoblasts in myogenic cell lines¹³¹ and long-term proliferating cells¹³². However, evidence for such effects on freshly isolated satellite cells (i.e., not passaged) are minimal at best¹³³ (and Yablonka-Reuveni, unpublished data).

Altogether, claims for adult stem cell plasticity can be made only when changes in the extracellular environment affect the genetic profile in a way that causes a stem cell to assume a lineage fate other than the expected one⁵³. Therefore, it is difficult to infer from studies with cell lines about properties of bona fide stem cells as cell lines do not respond to extracellular signals or express master regulator genes at levels that the bona-fide stem cells do. Importantly, in “native” multipotent stem cells, changes in extracellular signaling affect the regulation of master gene expression, and this reprogramming directs stem cells to acquire one out of several possible lineage fates. In contrast, many studies with cell lines show cell fate switches based on experimental manipulations intended to: (i) overexpress genes that are not part of the endogenous gene expression repertoire (e.g., forced expression of MyoD in nonmyogenic cells can dictate myogenic differentiation), or (ii) underexpress endogenous genes (e.g., by introducing dominant negative or inhibitory RNA constructs). Such experimentally-induced gene reprogramming in cell lines does not necessarily recapitulate normal in-vivo potency of cells to switch lineages upon changes in the extracellular signaling milieu.

Cells in primary myogenic cultures and in cultures emanating from isolated myofibers can express master regulators of several mesenchymal lineages

Claims for the plasticity of satellite cells were put forth again in recent studies aimed at unraveling whether muscle satellite cells are committed or multipotential stem cells. In view of earlier studies with cells lines, the effect of BMPs (BMP- 4, -7 or -2) as osteogenic inducers and of adipogenic inducers (methyl-isobutylxanthine, dexamethasone, indomethacin and insulin or a natural activator of PPAR γ (γ -linolenic acid) was assessed in cells emanating from single fibers and satellite-cell derived primary myogenic cells^{9,11}. In

both studies, cells within cultures emanating from single myofibers were shown to express osteogenic and adipogenic markers depending on the supplemented inducer.

In the study by Asakura and colleagues⁹, cultures were maintained in Ham's F10-based growth medium supplemented with 20% fetal bovine serum and FGF2, a commonly used approach for amplifying myoblasts isolated from the muscles of postnatal and young mice¹³⁴. In our hands, such F10-based growth medium promotes robust proliferation of nonmyogenic cells in primary myogenic cultures from adult and old mice¹³⁵. We further demonstrated that when individual myofibers are cultured in F10-based serum-rich/mitogen-rich medium, there was a drastic increase in the number of nonmyogenic cells and a drastic increase in the number of cells undergoing adipogenesis compared to cultures maintained in Dulbecco's Modified Eagle's Medium (DMEM)-based growth medium (unpublished results). Moreover, clonal analysis of cells associated with isolated myofibers revealed a higher number of nonmyogenic clones when cells were cultured in F10 medium. Notably, myogenic clones supplemented with F10 medium retained their myogenic potential and did not enter adipogenesis or other mesenchymal differentiation programs. In view of these results, we believe that myogenic cell preparations, even those derived from isolated myofibers, may at times carry along some nonmyogenic cells.

The reported spontaneous (i.e., without adding lineage inducers to the medium) adipogenic differentiation in single myofiber cultures and in clones derived from single myofibers^{10, 2} may, to some extent, be explained by the contribution of nonmyogenic cells that are associated with isolated myofibers. While we did not detect the presence of "contaminating" endothelial or hematopoietic cells in the freshly isolated myofibers when antibodies specific for these cell types were used (i.e., CD31 and CD45, amongst others¹²), we cannot rule out the possibility that some interstitial cells were co-cultured with the myofibers (but were not associated with the myofibers themselves). Clones derived from individual myofibers that contained both myogenic and adipogenic cells could reflect dual potential of satellite cells. However, although we were able to identify such clones (at a low frequency), careful examination of such clones during the first 3 days after cloning indicated that there were two growth foci, suggesting that two cells have established the apparent mixed clones.

Effect of age on fat accumulation in myofiber cultures

In advanced old age, lipid is redistributed from fat depots to muscle, bone marrow and other tissues. Indeed, myofibers of old rodents display at times increased level of fat accumulation^{13,136}. In view of this age-associated increase in fat accumulation and of our findings that cells emanating from preparations of isolated myofibers contain both myogenic and adipogenic progenitors, we aimed at further dissecting the relative contribution of the two distinct cell types in myofiber cultures isolated from old age. Accordingly, we analyzed parallel cultures derived from individual extensor digitorum longus (EDL) myofibers isolated from 6- and 26-month-old C57BL/6 mice that were maintained in our standard rich growth medium (G. Shefer and Z. Yablonka-Reuveni, unpublished results). Cultures were analyzed at one and two weeks after myofiber isolation, leading to the following conclusions:

- i. Some Pax7⁺ (myogenic) cells and myotubes in both adult and aging myofiber cultures exhibited similar low-level fat accumulation. The displayed minuscule droplets of fat (identified by oil red O staining) were related to the cells' metabolism but were not indicative of adipogenic differentiation, as cells did not demonstrate upregulation of adipogenic master regulators.

- ii. Nonmyogenic cells that differentiate into adipocytes containing large lipid vesicles (stained intensely with oil red O) were present in cultures from both adult and aging mice.
- iii. The number of myogenic cells (Pax7⁺ or MyoD⁺) emanating from individual myofibers was drastically reduced in myofiber cultures obtained from old mice²¹. Due to this decline in myoblast number, the frequency of myogenic cells compared to nonmyogenic/adipogenic cells was far reduced in myofiber cultures derived from aged animals.

The increased frequency of adipocyte producing cells in myofiber cultures, together with the decline in the abundance of myogenic cells, may reflect a physiologically relevant process that accounts, at least in part, for the age-linked enhanced adiposity of muscles. An earlier study proposed that myoblasts themselves acquire an increased adipogenic potential with age, based on the observation that myogenic cultures prepared from old animals displayed increased adipogenic potential when treated with an adipogenic inducing cocktail¹³⁶. However, the possibility that the cultures had an increased number of nonmyogenic cells even before the supplementation of adipogenic-inducing cocktail was not considered in the latter study. Presence of such cells could certainly lead to apparent enhanced adiposity in myogenic cultures since the nonmyogenic cells could be the contributors of RNA coding for the enhanced gene expression of adipogenic transcription factors. In our hands, myogenic cells never displayed elevated expression of adipogenic genes (regardless of donor muscle age), even when an inducing cocktail was added to primary myogenic cultures.

Do satellite cells possess mesenchymal plasticity?

In the previous section we detailed studies with primary myogenic cultures or with cultures emanating from isolated myofibers and further elaborated on the possibility that the contribution of additional cells as founders of the nonmyogenic cells could not be ruled out, even in the case of myofiber cultures. Nevertheless, one cannot entirely overlook the potential of satellite cells themselves to enter adipogenesis due to the following findings. First, adipogenic differentiation occurs spontaneously in myofiber cultures without introducing any chemical inducers^{10,12}. This adipogenic differentiation program seems to be a “default” secondary program in myofiber cultures maintained in plates coated with Matrigel (a basement membrane-like substratum) and supplemented with mitogen-rich/serum-rich DMEM-based medium¹². Under these culture conditions, satellite cells are activated, migrate off the fiber, proliferate and descendants fuse to form multinucleated myotubes. In parallel to myogenesis, adipogenic differentiation also takes place^{10, 2}. Nonmyogenic cells containing many small fat droplets (i.e., multilocular cells) emerge within the first week in culture and exhibit gradual increase in the accumulated fat, with time droplets merge to form a large fat deposit occupying most of the cells’ cytoplasm (i.e., unilocular cells)¹². These cytological changes are accompanied with the upregulation of transcription factors associated with adipogenesis, culminating in gene expression profile characteristic of the mature, terminally differentiated adipocyte^{12,13}. This precisely coordinated adipogenesis is much different from reports on induction of fat accumulation in myogenic cultures using chemical inducers^{11,136}. In the latter cases the myoblasts and myotubes themselves can exhibit accumulation of small fat droplets and it is unclear whether a true adipogenesis takes place.

Second, fat-containing cells (identified by their strong reaction with oil-red-O) do appear sometimes in close association with their parent myofiber, and this intimate contact suggests that the cells originated from the satellite cells niche and not from cells co-isolated from myofibers^{9,10}. This presence of fat-containing cells on myofibers was further enhanced in

myofibers that rapidly lost their integrity during initial cultures days or when isolating myofibers from mice of old age (our unpublished results).

Third, and most important, the maximum number of clones (myogenic and nonmyogenic clones combined) derived from an individual myofiber of adult muscle never exceeded the number of satellite cells on individual myofibers as determined by Pax7 immunostaining¹². This profile suggests that satellite cells were the origin of both myogenic and nonmyogenic clones, as it would otherwise be expected that, at least in some cases, the number of clones would exceed the maximal number of satellite cells as quantified in fixed freshly-isolated myofibers.

It is noteworthy that characteristics of cells producing the adipogenic cells are very much like those of mesenchymal stem cells¹². As observed in our studies, the nonmyogenic clones, which contained mature adipocytes, displayed additional cells expressing α -smooth muscle actin and nestin¹². This cell composition seems to mirror characteristics of mesenchymal stem cell progeny^{137,138}. The ratio between the myogenic and nonmyogenic clones suggested random distribution, and we thus proposed that satellite cells do possess mesenchymal plasticity and the decision to enter myogenesis or the MAD program is perhaps stochastic in our culture conditions, but is regulated in vivo by signals portrayed by the environment.

The idea of fate-switch between muscle and fat cells seems appealing, given the fact that accumulation of fat at the expense of muscle is a characteristic of maladies such as type-II diabetes, obesity, and myodystrophies such as Duchenne muscular dystrophy (DMD) as well in normal aging^{136,139–144}. An interplay between the myogenic and adipogenic lineages takes place during embryogenesis when the dermomyotome establishes the myogenic lineage¹⁴⁵, whereas somitic cells facing the notochord develop into mesenchyme and give rise to loose migratory cells and to adipogenic progenitors¹⁴⁶. Furthermore, studies of mice lacking p190-B RhoGAP, a modulator of Rho GTPase and IGF1 signaling, showed that embryo-derived fibroblasts obtained from these null mice underwent myogenesis at the expense of adipogenesis, a phenomenon not seen in wild type cells¹⁴⁷. Such studies of embryonic processes suggest that homeostasis between muscle and adipose tissues may be regulated at the stem cell level.

New experimental approaches are required to unequivocally determine if all or some satellite cells are multipotential, or at least have dual myogenic-adipogenic differentiation potential. Such cells may represent less committed satellite cells that retain a broader differentiation breadth. Alternatively, some cells in the satellite cells niche may be true mesenchymal stem cells that were able to transit into this niche from the intersitium or the circulation (see Fig. 7 in ref 12 for a proposed model). The idea that bone-marrow derived cells can be mobilized to the satellite-cell niche was supported in several studies^{81,86}. Another study, however, in which parabiosis was used, did not support this notion⁹⁴.

Critically, the phenomenon of satellite cell differentiation into mesenchymal alternative cell types (i.e., MAD program) needs to be investigated in vivo. Studying satellite cell plasticity in vivo is a challenging and not readily feasible task. A precedent to such in vivo experimentations is a recent study of salamander limb regeneration¹⁴⁸. In the latter study, progeny of satellite cells were expended, labeled in culture, and then injected intramuscularly before limb amputation. The injected cells were able to adopt nonmyogenic fate in the regenerating area (blastema), in the epidermis, and within newly formed cartilage tissue. As pointed by the authors, the observed multipotency of satellite cell progeny does not directly address the question of whether activated satellite cells adopt divergent fates without in vitro expansion. In order to investigate the lineage fate that satellite cells can take

in vivo, satellite cells will need to be isolated and labeled immediately without further culturing, and rapidly introduced back to the host tissue. Without such in vivo labeling, conclusions on satellite cells producing other cell types will remain speculative.

An example of in vivo de-regulation between mesenchymal lineages is the ectopic muscle ossification that occurs in fibrodysplasia ossificans progressive. This ossification is thought to result from overexpression of BMP4¹⁴⁹ that may affect satellite cells in vivo in a similar manner to its action when added to myogenic cultures and force the cells to divert into osteogenic differentiation path^{9,11}. However, this disease can be also attributed to abnormal pericyte differentiation, especially as pericytes were already shown to give rise to osteogenic cells both in culture and in vivo^{102,103}.

Concluding remarks

Figure 1 depicts the two main possible ways for interpreting the emergence of cells with an alternative mesenchymal phenotype in myofiber cultures. In our myofiber and clonal culture conditions, the alternative differentiation is adipogenesis. In the multipotent path, common satellite cells can give rise to different mesenchymal lineages (myogenic and nonmyogenic), depending on local physiological cues. In the unipotent path, satellite cells are not identical and vary in their potential. Furthermore, it is possible that nonmyogenic cells are contributed by cells that are tightly associated with the myofibers but not residing in the satellite cell niche. Additional cell culture and in vivo studies are required for reaching a consensus as to whether or not satellite cells possess a broader mesenchymal potential that goes beyond their well-established myogenic potential. Most likely, the development of mouse models with exclusive expression of fluorescent tag by satellite cells would permit tracing the fate of single cells in their native niche and in alternative tissue environments. Satellite cells were proven to be heterogeneous at the molecular level and this property may indeed reflect their potential for different lineage-fate preferences in response to different extracellular networks of signals. Alternatively, the apparent multipotency of satellite cells may actually be contributed by multipotential mesenchymal stem cells, intimately associated with muscle fibers or recruited to their vicinity in time of need. Pericytes from the capillary networks, which are tightly associated with myofibers, also need to be considered as a potential source for the mesenchymal alternative differentiation (MAD) seen in satellite cells cultures. It is attractive to consider the possibility that a broader mesenchymal potentiality of satellite cells is reminiscent of the plasticity they possess in amphibians¹⁴⁸. Perhaps when submitted to stress-inducing conditions such as highly mitogenic growth medium²¹ or high oxygen level¹⁰, mammalian satellite cells can show properties they have lost upon evolutionary specialization.

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FIGURE 1.

Adipogenic and myogenic cells present in single myofiber cultures either (1) derived from a single founder cells with a dual differentiation potentiality giving rise to myogenic and nonmyogenic cells (multipotent path) or (2) each is derived for different founder cells – one destined to give rise to myogenic progeny and another destined to give rise to nonmyogenic progeny (unipotent path).