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Placenta-derived mesenchymal stromal cells and their exosomes exert therapeutic effects in Duchenne muscular dystrophy

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ABSTRACT

Duchenne muscular dystrophy (DMD) is a degenerative lethal, X-linked disease of skeletal and cardiac muscles caused by mutations in the dystrophin gene. Cell therapy using different cell types, including mesenchymal stromal cells (MSCs), has been considered as a potential approach for the treatment of DMD. MSCs can be obtained from autologous sources such as bone marrow and adipose tissues or from allogeneic placenta and umbilical cord. The safety and therapeutic impact of these cells has been demonstrated in pre-clinical and clinical studies and their functions are attributed to paracrine effects that are mediated by secreted cytokines and extracellular vesicles. Here, we studied the therapeutic effects of placenta-derived MSCs (PL-MSCs) and their secreted exosomes using mouse and human myoblasts from healthy controls, Duchenne patients and mdx mice. Treatment of myoblasts with conditioned medium or exosomes secreted by PL-MSCs increased the differentiation of these cells and decreased the expression of fibrogenic genes in DMD patient myoblasts. In addition, these treatments also increased the expression of utrophin in these cells. Using a quantitative miR-29c reporter, we demonstrated that the PL-MSC effects were partly mediated by the transfer of exosomal miR-29c. Intramuscular transplantation of PL-MSCs in mdx mice resulted in decreased creatine kinase levels. PL-MSCs significantly decreased the expression of TGF-β and the level of fibrosis in the diaphragm and cardiac muscles, inhibited inflammation and increased utrophin expression. In vivo imaging analyses using MSCs labeled with gold nanoparticles or fluorescent dyes demonstrated localization of the cells in the muscle tissues up to 3 weeks post treatment. Altogether, these results demonstrate that PL-MSCs and their secreted exosomes have important clinical applications in cell therapy of DMD partly via the targeted delivery of exosomal miR-29c.

1. Introduction

Duchenne muscular dystrophy (DMD) is a recessive, fatal, X-linked disease and the most common form of muscular dystrophy [1]. DMD is caused by mutations in the dystrophin gene that can be either spontaneous or inherited [2]. The dystrophin protein plays a critical role in the maintenance, integrity and normal functions of muscle cells, and loss of this protein leads to progressive muscle degeneration [3]. Under normal conditions, muscle degeneration activates muscle stem cells (satellite cells), that eventually differentiate into mature muscle cells and contribute to tissue regeneration [4]. Recent studies indicate that dystrophin is also essential for the asymmetric division of satellite cells and lack of functional protein interferes with the regenerative capacity of these cells [5,6]. Thus, muscle differentiation and regeneration are impaired in

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DMD, and this results in replacement of the degenerated muscles with fibrotic and fat tissues [1].

Various therapeutic approaches for treating DMD have been explored in pre-clinical and clinical studies. These include promotion of muscle regeneration [7], anti-inflammatory and anti-fibrotic agents [2,8–10], exon-skipping antisense oligonucleotides [11], and other therapies to restore dystrophin expression [12]. In addition, stem cell-based therapy, using transplantation of satellite cells [13], myogenic progenitors, mesoangioblasts [14], pericytes [15] and mesenchymal stromal cells (MSCs) [16,17] has been also explored for the treatment of DMD.

MSCs are mesoderm-derived cells that can be obtained from autologous or allogeneic sources [18]. These cells can differentiate into adipogenic, chondrogenic and osteogenic lineages [19] in addition to some degree of myogenic differentiation upon engraftment in muscle tissues [20] or in the presence of DNA demethylation agents [21].

MSCs express low levels of MHCII, and are therefore considered non-immunogenic [22]. MSCs from different sources have been widely explored for the treatment of various inflammatory and degenerative disorders [23]. In DMD animal models, MSC transplantation exerted some anti-inflammatory and promoted regenerative activity in damaged muscles [24]. The beneficial effects of MSCs have been attributed to the secretion of a variety of bioactive molecules such as growth factors, cytokines and chemokines that exert immunomodulatory effects and provide trophic support. In addition, recent studies demonstrated that MSCs secrete a large number of extracellular vesicles [25] that can also mediate the various effects of the cells by playing important roles in intercellular communication.

In this study, we analyzed the effects of MSCs and secreted exosomes on DMD muscles using both in vitro and in vivo models. We found that MSCs derived from placenta (PL-MSCs) increased the differentiation of human muscle cells from DMD patients and identified exosomal miR-29 as a mediator of this effect. Moreover, PL-MSCs and their secreted exosomes (PL-exosomes) also exerted a therapeutic impact in mdx mice, decreasing CK levels, inhibiting fibrosis and inflammation while increasing utrophin expression.

2. Results

2.1. Conditioned medium from PL-MSCs increases myoblast differentiation

PL-MSC effects were examined on the differentiation of C2C12 cells and cultured human myoblasts. Muscle cells were maintained in a medium containing 2% horse serum for 4–6 days to allow cell differentiation and fusion. We employed co-culture systems consisting of transwell plates with a 1-μm filter which only allows the transfer of secreted factors and exosomes. C2C12 cells that were co-cultured with PL-MSCs exhibited a significant increase in cell fusion (Fig. 1a) and expression of the differentiation markers, myosin heavy chain (MyHC) and myogenin (Fig. 1b and c). Some increase in MyHC expression was also induced by umbilical cord-derived MSCs (UC-MSCs), whereas smaller effects were observed in C2C12 cells co-cultured with adipose tissue-derived MSCs (AD-MSCs) and no significant effect by bone marrow-derived MSCs (BM-MSCs).

Myoblasts from mdx mice have been reported to exhibit impaired differentiation capabilities [26]. We therefore examined the effects of the PL-MSCs on the differentiation of human myoblasts derived from healthy donors and DMD patients. The DMD-myoblasts exhibited a lower degree of cell differentiation as indicated by the decreased MyHC expression (Fig. 1d and e). Treatment of the human myoblasts with conditioned medium of PL-MSCs using a similar co-culture system, increased the expression of MyHC (Fig. 1f and g) and troponin (Fig. 1g). Also for these cells, the effects of the PL-MSCs on MyHC expression were larger than that of the co-cultured BM-MSCs (Fig. 1h).

2.2. Exosomes secreted by PL-MSCs promote myoblast fusion and differentiation

MSCs have been reported to exert their paracrine effects by both secreted cytokines and exosomes [25]. To determine the role of MSC-derived exosomes in the PL-MSC effects, we isolated exosomes from these cells as previously described [27,28]. The isolated exosomes were analyzed for the expression of CD63 and CD9 using Western blot analysis (Fig. S1A), and the amount of the exosomes was determined using ELISA of CD68 antibodies (Fig. S1B). We first demonstrated the transfer of exosomes from PL-MSCs (PL-exosomes) to myoblasts using ImageStreamX analysis. In these studies, PL-exosomes labeled with CellTracker Red, were added to C2C12 cells (Fig. 2a) or human myoblasts (Fig. 2b) and the muscle cell fluorescence was determined 12 h later. The PL-exosomes were efficiently internalized by the myoblasts and accumulated in the cells. Similar results were obtained using confocal microscopy (Fig. 2c). The PL-exosomes significantly increased the fusion of the myoblasts, as demonstrated by confocal microscopy (Fig. 2d), similar to the effects of the co-cultured PL-MSCs (Fig. 1a). In addition, the PL-exosomes exerted a similar effect to that of the conditioned medium and increased expression of MyHC (Fig. 2e and f) and troponin (Fig. 2f) in the treated myoblasts. In contrast, BM-exosomes did not exert a significant effect on the expression of MyHC or myogenin in the treated DMD cells (Fig. 2f).

2.3. The effects of PL-MSCs on myoblast differentiation are mediated via the transfer of exosomal miR-29c

To delineate the mechanisms involved in the effects of the PL-MSCs, we analyzed the expression of specific miRNAs that are associated with myogenic differentiation in the PL-exosomes. Since BM-MSCs and their secreted exosomes did not exert significant effects on the differentiation of the human DMD myoblasts, we focused on the miR-29 that is highly expressed in PL-exosomes compared with BM-exosomes (Fig. 3a). Moreover, miR-29 has been reported to be downregulated in muscle of mdx mice compared with healthy controls, and was associated with various pathological pathways in DMD [29,30]. We found that the expression of miR-29 was also significantly decreased in muscle cell cultures from DMD patients compared with those of healthy donors (Fig. 3b). Low levels of the miR-29 family were associated with acquisition of DMD phenotype [29] and overexpression of these miRNAs promoted differentiation of myoblasts derived from mdx mice. We therefore hypothesized that the transfer of miR-29 by the PL-exosomes may mediate the increased differentiation of the DMD-derived myoblasts induced by the PL-MSCs. We focused on miR-29c since this miRNA appears to exert the most significant therapeutic effect in DMD mouse models [31]. To demonstrate the transfer of exosomal miR-29c by the PL-MSCs into myoblasts we employed a miR-29c reporter tagged to luciferase that enables the quantification of transferred miRNAs as was previously reported for miR-124 [27]. Myoblasts were transduced with a lentiviral vector expressing the miR-29c luciferase reporter and transfected with a miR-29c mimic or co-cultured with PL-MSCs using transwell plates with 1-μm filters. BM-MSCs were employed as a negative control. As presented in Fig. 3c, myoblasts expressing the miR-29c reporter and either overexpressing miR-29c mimic or co-cultured with PL-MSCs exhibited decreased luciferase activity, indicating that miR-29c was transferred by the PL-MSCs to the myoblasts. Similar