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Myogenic induction of adult and pluripotent stem cells using recombinant proteins

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ABSTRACT

Met Activating Genetically Improved Chimeric Factor 1 (Magic-F1) is a human recombinant protein, derived from dimerization of the receptor-binding domain of hepatocyte growth factor. Previous experiments demonstrate that in transgenic mice, the skeletal muscle specific expression of Magic-F1 can induce a constitutive muscular hypertrophy, improving running performance and accelerating muscle regeneration after injury.

In order to evaluate the therapeutic potential of Magic-F1, we tested its effect on multipotent and pluripotent stem cells. In murine mesoangioblasts (adult vessel-associated stem cells), the presence of Magic-F1 did not alter their osteogenic, adipogenic or smooth muscle differentiation ability. However, when analyzing their myogenic potential, mesoangioblasts expressing Magic-F1 differentiated spontaneously into myotubes. Finally, Magic-F1 inducible cassette was inserted into a murine embryonic stem cell line by homologous recombination. When embryonic stem cells were subjected to myogenic differentiation, the presence of Magic-F1 resulted in the upregulation of Pax3 and Pax7 that enhanced the myogenic commitment of transgenic pluripotent stem cells.

Taken together our results candidate Magic-F1 as a potent myogenic stimulator, able to enhance muscular differentiation from both adult and pluripotent stem cells.

1. Introduction

Muscle diseases are a group of heterogeneous disorders, due to structural or functional abnormalities in the skeletal muscle, which then lead to muscular waste and force decrease. Although muscle diseases still lack an effective therapy, several novel strategies are entering into clinical trials, including gene replacement, exon skipping, stem cell therapies and treatments to induce muscle hypertrophy [1,2]. Previous studies have been shown that inducing hypertrophy (through loss of myostatin or IGF upregulation) ameliorates the muscular regeneration and attenuates the severity of the disease in animal model of muscular dystrophy [3,4]. Therefore, the possibility to induce muscular hypertrophy has also a potential clinical implication for the treatment of both genetic and acquired muscle diseases [5]. We previously showed that Magic-F1 (Met-Activating Genetically-Improved Chimeric Factor 1) is a recombinant protein able to induce a beneficial effect in dystrophic mice, due to the constitutive hypertrophy, that partially rescues the muscle phenotype [6]. Magic-F1 is derived from a repetition of the high-affinity receptor-binding domain of human hepatocyte growth factor (HGF). Thanks to its peculiar structure, Magic-F1 binds Met, HGF receptor, and elicits a selective pattern of biological responses, enhancing the myogenic differentiation process,

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protecting the muscle precursors against apoptosis, without stimulating cell proliferation.

Mesoangioblasts (MABs), vessel-associated progenitors, have been described in murine, canine and human skeletal muscle [7–9]. When transplanted, they are able to cross the blood vessels and differentiate into skeletal muscle *in vivo* [7,8]. A phase I/II clinical trial is ongoing to test the safety of intra-arterial allogenic transplantation of MABs in Duchenne muscular dystrophy patients. Moreover, the technology of reprogramming adult somatic cells into induced pluripotent stem cells (iPSC) [10,11] has opened a wide range of future possibilities in terms of personalized medicine. Patient-specific iPSC could be corrected and induced to differentiate into skeletal muscle progenitors, offering an autologous and expandable source for cell transplantation. To reach this aim, different protocols have been described for the differentiation of murine pluripotent stem cells towards myogenic progenitors, by overexpressing the paired-box transcription factors Pax3 and/or Pax7 or by particular culture conditions [12–16]. Pax3-induced cells, derived from embryonic stem cells (ESCs) and isolated as PDGFRα^{+}/Flk1^{-} population, engrafted in skeletal muscles of dystrophic mice, improving contractility [12,13]. The same results were obtained from iPSC, by overexpressing Pax7 [14]. Furthermore, Pax7-positive satellite-like cells, derived from ESCs or iPSC differentiation, have been isolated by fluorescence-activated cell sorting (FACS), using a novel antibody (SM/C-2.6). These muscle progenitors were able to differentiate into skeletal muscle fibers both *in vitro* and *in vivo* [15,16].

We recently reported that Magic-F1 is expressed in developing tissues of mesenchymal origin in Magic-F1 transgenic mice, where also Pax3 is expressed [17]. The fact that Magic-F1 could be responsible of muscular hypertrophy, cooperating with Pax3 signal pathway, in skeletal muscle precursor cells encouraged us to explore the myogenic potential of Magic-F1 in adult and embryonic stem cells.

Therefore here we investigated novel strategies to improve myogenic differentiation in adult and pluripotent stem cells with recombinant proteins, which in principle can be used as adjuvant compounds for a plethora of skeletal muscle regenerative applications.

2. Materials and methods

2.1. Cell cultures

C2C12 cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) high glucose supplemented with 2 mM glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin, 1 mM sodium pyruvate and 10% FBS (all from GBCO).

MABs have been isolated, established and expanded as previously described [18,19]. The growing medium contains 20% Fetal Bovine Serum (FBS), 2 mM glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin and 1 mM sodium pyruvate.

Mouse ESCs KH2 were grown on feeder layer of mitotically inactivated Mouse Embryonic Fibroblasts (MEF) or feeder free, on gelatin-coated dish. Cells were passaged every 2–3 days and grown in medium containing 20% FBS, recombinant mouse leukemia inhibitory factor (1000 U/ml), MEK inhibitor (1 μM, PD0325901 Axon Medchen) and GSK inhibitor (3 μM, Chir99021 Axon Medchen).

2.2. Plasmids

Magic-F1 cDNA was cloned in pTRIPZ lentiviral vector (Thermo Scientific). Magic-F1 and GFP cDNA were cloned in pBS31 vector. The plasmids were sequenced using the Big Dye Terminator V3.1 kit (ABI) and prepared by following standard procedures of PureLink HiPure Plasmid Filter Maxiprep Kit (Invitrogen).

2.3. Viral production and infection

293 cells were transfected with 6 μg of packaging plasmid (psPAX2, #12260 Addgene), 2 μg of envelope plasmid (pMD2G, #12259 Addgene) and 4 μg of transfer vector (pTRIPZ-Magic-F1 or pWPT-nlsLacZ, #12261 Addgene), using Lipofectamine 2000 (Invitrogen), following manufacturer's protocol. Virus containing supernatants were harvested 36–48 h post-transfection, filtered to remove cell debris and added into the well, where the day before C2C12 cells or MABs were plated. Medium was replaced 24 hafter infection and after 48 h the antibiotic was added to select the infected cells.

2.4. ESCs inducible cell lines

KH2 transgenic lines have been generated following manufacturer's instruction (Gene targeting kit, Thermo Scientific). Briefly, KH2 cells, which contain two FRT-sites and an ATG-less, promoterless hygromycin cassette, were nucleofected with 15 μg of pBS31-GFP or -Magic-F1 (carrying the cDNAs, the PGK promoter and the ATG start codon) and 7.5 μg of pCAGG-Flp plasmid (for the flippase enzyme expression), in a Nucleofector™ 2b Device (Lonza), following the instructions of Mouse ES cell Nucleofector® Kit (Lonza). Homologous recombination allowed the cDNA insertion, conferring hygromycin resistance. After 24 h, ESCs were re-plated on hygromycin-resistant MEF, with 150 μg/ml of hygromycin. Expression of the transgenes was induced adding doxycycline (1 μg/ml) to the medium.

2.5. Mesodermal differentiation assays

Myogenic differentiation of C2C12 cells was obtained by serum starvation in differentiation medium (DMEM with 2% heat-inactivated horse serum (HS), 2 mM glutamine, 1 mM sodium pyruvate), in presence of doxycycline (1 μg/ml) when indicated. Myotube formation was evaluated by immunofluorescence analysis for sarcomeric myosin heavy chain (MyHC). Fusion index was calculated counting the nuclei inside the myotube, divided by the total number of nuclei. At least 4 different pictures for each condition and each time point were counted, using Image J software (http://rsbweb.nih.gov/ij/).

The MABs obtained were differentiated into adipocytes, osteocytes, smooth muscle, and skeletal muscle following protocols already present in the lab [18,19]. To test their myogenic potential, MABs were differentiated alone or in co-culture with nLacZ-C2C12 cell in DMEM containing 2% HS. After one week, immunofluorescence analysis for MyHC and X-gal staining were performed to check myotubes formation and label the C2C12 nuclei. The fusion index has been calculated comparing the immunofluorescence and the bright field images, counting the MABs nuclei (negative for X-gal staining) inside the myotubes, divided by the total number of MABs nuclei.

KH2 cells were detached with trypsin, counted and diluted at 10000 cells/ml in differentiation medium (DMEM high glucose with 0.1 mM nonessential amino acids, 100 μg/ml streptomycin and 100 U/ml penicillin, 0.1 mM 2-mercaptoethanol, 5% HS and 10% FBS). 1000 cells were plated into each well of an ultralow attachment 96-wells plate (Corning). Forced aggregation was induced by centrifugation at 1600 rpm for 6 min. After 48 h the medium was changed, adding doxycycline (1 μg/ml). EBs were collected at day 0, 3, 5 and 7 for RNA analysis. At day 7, EBs were collected and analyzed by FACS Canto or plated in fibronectin-coated 48 well

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