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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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plate, to induce the myogenic differentiation in DMEM containing 5% HS. Myogenic differentiation was evaluated as number of myotubes per well.

2.6. LacZ staining

Cells were washed in PBS and with a fixing solution, containing glutaraldehyde, EDTA and $MgCl_2$. Wells were washed with washing buffer (with NP40 and deoxycholate). The LacZ staining was performed at 37 °C overnight, adding a solution with X-gal, potassium ferrocyanide and potassium ferricyanide.

2.7. Immunofluorescence analysis

Immunofluorescence analysis was performed as previously described [6]. Cells were incubated with primary antibodies against MyHC (MF20, hybridoma bank, University of Iowa), Nanog (14-5761-80, eBioscience), Oct4 (sc-8628, Santa Cruz) and Sox2 (ab 5603, Millipore) or isotype control and then with the appropriate FITC- or TRITC-conjugated secondary donkey antibodies (1:500, Invitrogen). Images were taken by Eclipse Ti microscope (Nikon) and merged by Image-Pro Plus 6.0 software.

2.8. Western blot analysis

Western blot was performed as previously described [6]. Membranes were incubated with antibodies against: HGF (sc-1357, Santa Cruz), Beta-Tubulin (05-661, Millipore), GFP (ab-5450, Abcam) and later with donkey horseradish peroxidase-coupled secondary antibody (1:5000, Invitrogen). Specific bands were detected with Chemiluminescent Peroxidase Reagent and pictures were taken with GelDoc (Biorad).

2.9. RNA extraction and analysis

Total RNA was extracted using GeneElute Mammalian Total RNA Miniprep Kit (Sigma), following the manufacturer's protocol. After reverse transcription (SuperScriptTM III First-Strand Synthesis SuperMix kit, Invitrogen), Real-Time qPCR was performed, following the Syber Green Mix (Invitrogen) protocol, on real time system Realplex2 Master Cycler (Eppendorf) or on ViiA 7 Real-Time PCR system (Invitrogen).

Oct4 Fw 5″ – CCAGGCAGGAGCACGAGTGG – 3″
Oct4 Rv 5″ – CCACGTCGGCCTGGGTGTAC – 3″
Eomes Fw 5″ – AGAACCGTGCCACAGACCAA – 3″
Eomes Rv 5″ – TGGTCACAGGTTGCTGGACA – 3″
Mxl1 Fw 5″ – ACCACCAGGCCTGACAACCT – 3″
Mxl1 Rv 5″ – TGGGTGCACACCATACCACA – 3″
T-Bra Fw 5″ – GTCAGACCAAGATCGCTTCT – 3″
T-Bra Rv 5″ – GATCGCTTCTGTCAGACCAA – 3″
Pax3 Fw 5″ – TCCATCCGACCTGGTGCCAT – 3″
Pax3 Rv 5″ – TTCTCCAGCTCAGGCGTTG – 3″
Pax7 Fw 5″ – GCCTTCAACCACCTTCTGC – 3″
Pax7 Rv 5″ – ACTGTGCTGCCTCCATCTTG – 3″
Gapdh Fw 5″ – CGAGACCCCACTAACATCAAA – 3″
Gapdh Rv 5″ – CATTGCTGACAATCTTGAGTGA – 3″

2.10. FACS analysis

EBs were dissociated using Cell dissociation buffer enzyme free Hank's based (Invitrogen) counted and incubated with biotinylated anti-SM/C-2.6 antibody (kindly provided by Dr. Fukada) or with biotinylated Rat IgG isotype. After two washes in PBS, cells were incubated with APC-conjugated streptavidin. Samples were analyzed on Canto Flow cytometer (BD bioscience) or sorted with FACSAria Flow cytometer (BD bioscience). Sorted cells were plated on matrigel in growing medium and, once confluent, differentiate into myotube in DMEM 5% HS.

2.11. Statistical analysis

The results are expressed as average ± standard deviation (SD). When data distribution approximated normality and two groups were compared, a Student's t-test was used. When three or more groups were compared a two-way ANOVA was used. All statistical tests were performed via Prism software (GraphPad). Significance was set at $p < 0.05$ or $p < 0.001$.

3. Results

3.1. Generation and validation of inducible lentiviral vector for Magic-F1 expression

C2C12 were transduced with lentiviral particles carrying Magic-F1 cDNA and were differentiated into myotubes, in presence or absence of doxycycline. Immunofluorescence analysis for MyHC was performed at day 3, 5 and 7 (Fig. 1A). The efficacy of the inducible system and the expression of transgenic protein were verified by Western-blot analysis (Fig. 1B). In presence of Magic-F1, the myogenic differentiation was accelerated and C2C12 cells formed larger myotubes, with more nuclei compared to the control, as showed by fusion index analysis (Fig. 1C).

3.2. Effect of Magic-F1 expression on adult mesoangioblasts

Next we tested the effect of Magic-F1 expression on MABs, which were infected with lentiviral particle, for an inducible expression of the transgenic protein. The presence or absence of Magic-F1 did not alter their ability to differentiate towards adipocytes, osteocytes or smooth muscle (Fig. 2A). However, Magic-F1 expression was able to induce the differentiation of MABs into myotubes (Fig. 2A), even without the presence of myoblast cell line, normally required to stimulate the myogenic differentiation. When MABs were co-cultured with C2C12, Magic-F1 expressing cells better contribute to myogenic differentiation, as shown by their increased fusion index (Fig. 2B).

3.3. Generation of an inducible Magic-F1 or GFP transgenic ESC line

KH2 cells were nucleofected with targeting vectors containing Magic-F1 or GFP cDNA and cells, in which recombination has occurred, were selected with hygromycin. Induction of the transgene was achieved adding doxycycline in the medium and checked by Western Blot analysis (Suppl. Fig. 1A). Gene expression analysis, for self-renewal-controlling genes (Oct4, Sox2, Nanog, Tbx3, Rex1 and Stella), confirmed that the targeting did not alter their pluripotency (Suppl. Fig. 1B). The presence of Oct4, Nanog and Sox2 at protein levels was checked by immunofluorescence analysis on GFP KH2 and Magic-F1 KH2 ESCs (Suppl. Fig. 1C).

3.4. Effect of Magic-F1 expression on ESCs myogenic differentiation

ESCs were grown in feeder free conditions on gelatin-coated dishes and plated in ultralow attachment plate to generate embryoid bodies (EBs), after forced aggregation by centrifugation. At day 2, doxycycline was added to the medium and EBs were cultured for 7 days (Suppl. Fig. 2A). The RNAs isolated from Magic-F1 and GFP EBs were analyzed at different time points (day 0, 3, 5 and 7) for the expression of markers of primitive streak and early

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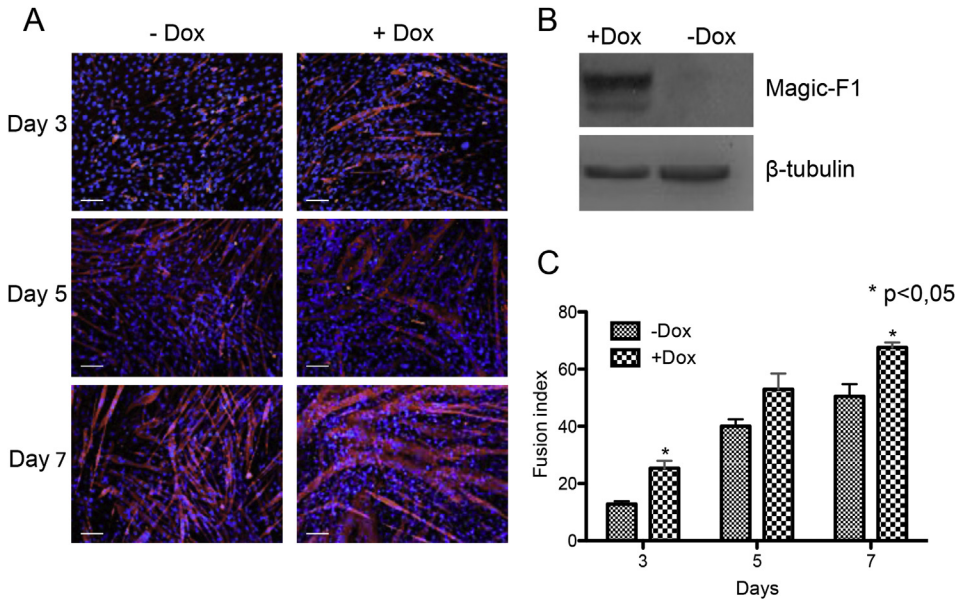

Fig. 1. Magic-F1 inducible lentiviral vector tested on C2C12. (A) Immunofluorescence analysis for MyHC expression on Magic-F1 C2C12 in absence (−Dox) or presence of doxycycline (+Dox) after 3, 5 and 7 days of culture in differentiation medium. Nuclei were stained with DAPI. Scale bar = 50 μm. (B) Western Blot analysis for Magic-F1, upon induction with doxycycline in Magic-F1 C2C12; β-tubulin was used as loading control. (C) Graph showing the fusion index of C2C12 cells differentiated in presence (+Dox) or in absence (−Dox) of Magic-F1 at day 3, 5 and 7; n = 5, *p < 0.05.

mesoderm differentiation (Eomes, Mixl1 and T-Bra), in order to confirm the transition from a pluripotent to a mesodermal state (Suppl. Fig. 2B). In presence of Magic-F1, the expression of these markers shows an up-regulation at day 3 of differentiation, if compared to the control. Moreover, when Pax3 and Pax7 expression was evaluated at day 5, 7 and 9 of differentiation, the Real Time PCR (qRT-PCR) analysis showed a higher expression of these paired-box transcription factors, when Magic-F1 was present, especially at day 7 (Fig. 3A). At day 7, EBs were plated onto fibronectin-coated plates. After EBs-adhesion, cells spread out and grow as a monolayer. To induce terminal differentiation, medium containing 5% of serum was added to the cells, as soon as they reached confluence. In presence of Magic-F1, the myogenic commitment of ESCs was enhanced, as shown by spontaneous myotubes formation (Fig. 3B), suggesting that Magic-F1 could increase the amount of muscle progenitors in differentiating EBs. The analysis of the number of MyHC positive myotubes per well confirmed the myogenic differentiation potential of the recombinant protein (Fig. 3C). In order to quantify and purely isolate these myogenic precursors from Magic-F1 induced EBs, SM/C-2.6

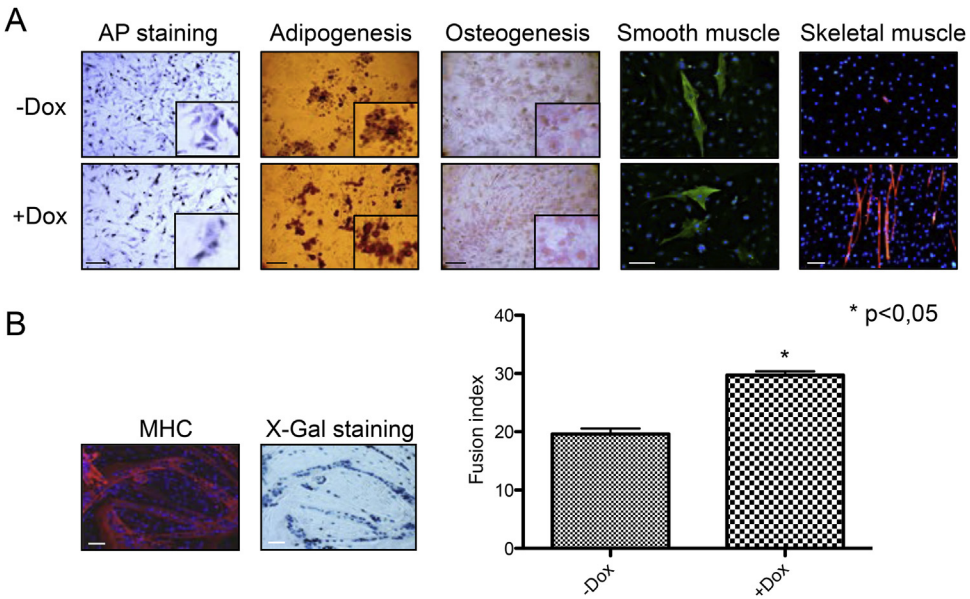

Fig. 2. Effect of Magic-F1 expression on adult mesoangioblasts. (A) Alkaline phosphatase staining of Magic-F1 transduced MABs and analysis of their differentiation abilities towards adipocytes, osteocytes, smooth muscle and myotubes in absence (−Dox) or presence of doxycycline (+Dox). Scale bar = 50 μm. (B) Myogenic differentiation of Magic-F1 transduced MABs in co-culture with nLacZ C2C12 cells: immunofluorescence for MyHC and X-gal staining were compared to calculate the fusion index, as the ratio of the number of X-gal negative nuclei inside the myotubes, versus the total number of X-gal negative nuclei Scale bar = 50 μm; n = 5, *p < 0.05.

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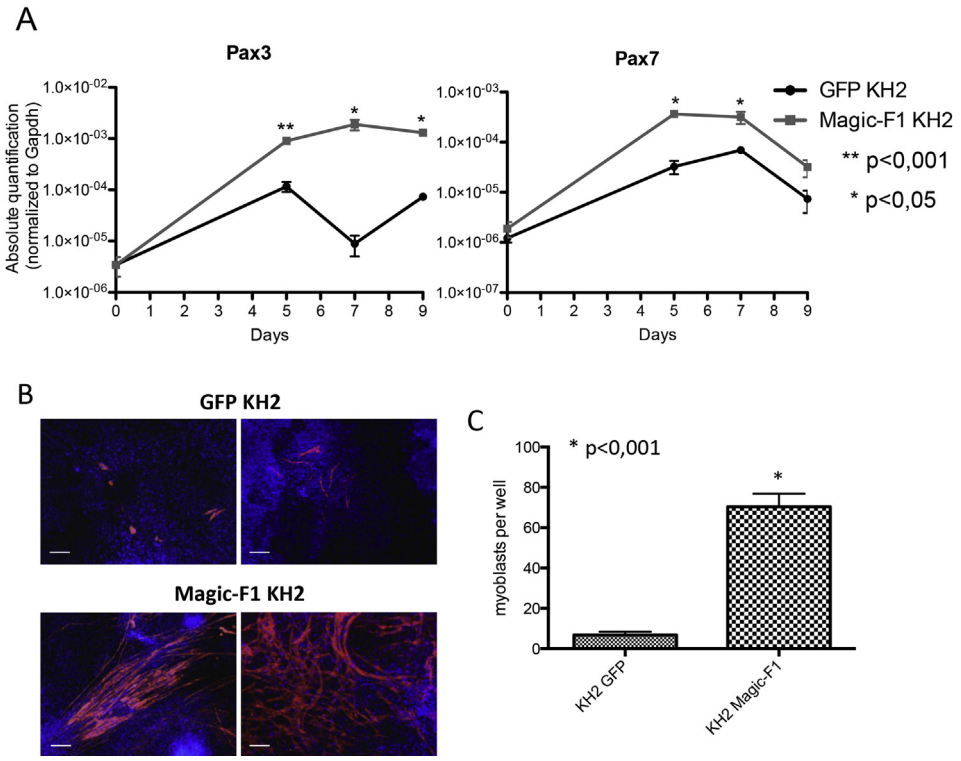

Fig. 3. Enhanced myogenic potential of Magic-F1 expressing ESCs. (A) qRT-PCR results for the expression of Pax3 and Pax7 in Magic-F1 and GFP-derived EBs in presence of doxycycline at day 0, 5, 7 and 9. Shown are Mean ± SD of absolute quantification for the indicated genes, normalized using Gapdh (logarithmic scale, n = 3, $^{*}p < 0.05$, $^{**}p < 0.001$). (B) Immunofluorescence analysis for MyHC showing myotubes formation in Magic-F1 or GFP KH2-derived EBs. Nuclei were stained with DAPI. Scale bar = 100 μm. (C) Quantification of MyHC positive myotubes per well in Magic-F1 or GFP KH2 derived-EBs, n = 3 $^{*}p < 0.001$.

antibody was used to detect satellite-like cells. Differentiated EBs were collected from the 96 wells on day 7 and stained for SM/C-2.6 antibody. FACS analysis showed that in presence of Magic-F1, SM/C-2.6 positive cells were detected on an average of 14%, differently from their control (~2%, Fig. 4A). Finally, SM/C-2.6 positive cells were also positive for myogenic markers, as Pax3, Pax7 and Myf5 (Fig. 4B) and able to fully differentiated in myotubes (Fig. 4C) as previously reported [15].

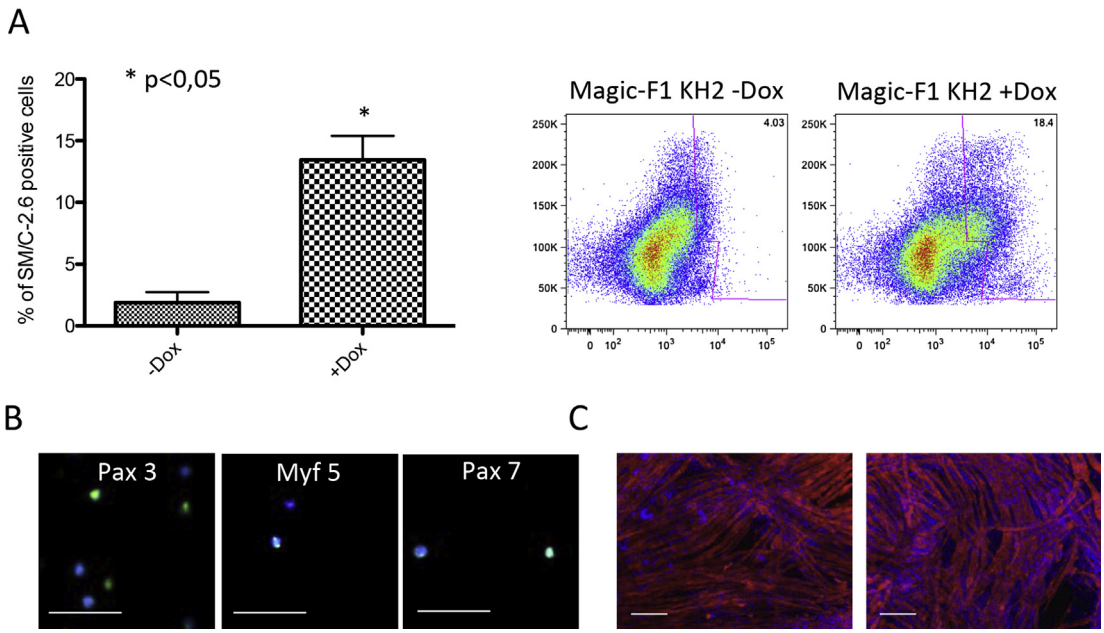

Fig. 4. Purification of SM/C-2.6 from ESCs subjected to myogenic differentiation. (A) Quantification of SM/C-2.6 positive cells by FACS on disaggregated EBs of Magic-F1 KH2 with or without doxycycline at day 7; n = 5, $^{*}p < 0.05$. Representative scatter plots showing the distribution of SM/C-2.6 positive cells are also reported. (B) Immunofluorescence analysis for Pax3, Pax7 and Myf5 in sorted SM/C-2.6 positive cells. Nuclei were stained with DAPI; scale bar = 50 μm. (C) Examples of immunofluorescence analysis for MyHC in SM/C-2.6 positive cells subjected to myogenic differentiation. Nuclei were stained with DAPI; scale bar = 100 μm.

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4. Discussion

HGF is a pleiotropic cytokine that plays a role in a variety of normal cellular processes. HGF activates a tyrosine kinase-dependent signaling cascade, after binding the proto-oncogenic Met receptor [20]. During embryogenesis, this cytokine is involved in the detachment of myogenic precursor cells from the lateral dermamyotome and their subsequent migration into the limb buds [21], while in the adult, the HGF-Met pathway is activated in muscle regeneration after injury, activating satellite cells proliferation [22]. However, HGF administration to muscle cells inhibits muscle differentiation both *in vitro* and *in vivo* [22,23]. To overcome this limitation we generated Magic-F1 as human HGF-derived protein and showed that differently from HGF, Magic-F1 activates AKT, but not ERK signaling pathway [6]; thus, the transgenic protein does not have any mitogenic activity. *In vivo* studies have demonstrated that, following cardiotoxin treatment, regenerating centrally nucleated fibers in Magic-F1 transgenic mice appear to have a greater cross-sectional area compared to wild-type animals. This can be explained by the enhanced differentiation potential of satellite cells, which indeed displayed an earlier differentiation program *in vitro* compared to cells isolated from wild-type mice [6].

In this work we looked into the effect of Magic-F1 during the differentiation of adult and pluripotent stem cells toward skeletal muscles. Lentiviral vectors were generated for an inducible expression of Magic-F1 and tested in C2C12 cells, a useful model for myogenic differentiation, where the expression of the transgenic protein accelerated the process of myotube formation.

We previously reported the presence of myogenic precursors, named mesoangioblasts, which differ from satellite cells and express pericyte markers [7–9]. We also speculated that MABs could be positively affected by Magic-F1 and actively participate to the muscle hypertrophy observed in transgenic mice [6]. Here we proved that indeed the recombinant protein was able to induce myogenic differentiation in MABs, in absence of C2C12 or satellite cells. These results in adult stem cells encourage us to study the effect of the transgenic protein in pluripotent stem cells. Several protocols have been proposed to generate skeletal muscle progenitors from ESCs. Unfortunately, the efficiency of these protocols is limited by the insufficient signals that induce patterning of the paraxial mesoderm. Due to this limitation, the majority of published protocols depend on forced Pax3 or Pax7 transgene expression [12–14] or on isolation of ES-derived satellite-like cells by SM/C-2.6 antibody [15]. We decided to generate transgenic ES cell line, where the expression of Magic-F1 or GFP could be induced by doxycycline. The new transgenic lines were differentiated towards skeletal muscle, adapting the protocol from Ref. [15]. In presence of Magic-F1, primitive streak markers (Mixl1, T and Eomes) showed an upregulation on day 3, while Pax3 and Pax7 on day 7, suggesting the ability of the transgenic protein to enhance the myogenic commitment of ESCs. This observation was confirmed by FACS analysis for SM/C-2.6, which showed a strong increase of positive cells in the Magic-F1 expressing EBs. Sorting for SM/C-2.6 allowed us to isolate myogenic progenitors, expressing satellite cells markers (Pax3, Pax7 and Myf5), and able to differentiate into myotube.

In conclusion, our results demonstrate that Magic-F1 is an engineered factor that provides pro-differentiative clues towards myogenic commitments in both adult and embryonic stem cells. This ability makes Magic-F1 a potential candidate for regenerative medicine and a valuable tool to counteract muscle wastage in muscular diseases, such as cachexia and muscular dystrophies.

Author contribution

IP designed, performed, analyzed experiments; IE, ALN, FR, EB, HG performed and analyzed experiments; MS analyzed experiments, drafted the manuscript and provided funding.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.07.022.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.07.022.

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