Tat-MyoD fused proteins, together with C2c12 conditioned medium, are able to induce equine adult mesenchimal stem cells towards the myogenic fate

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Tat-MyoD FUSED PROTEINS, TOGETHER WITH C2C12 CONDITIONED MEDIUM, ARE ABLE TO INDUCE EQUINE ADULT MESENCHIMAL STEM CELLS TOWARDS THE MYOGENIC FATE.

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Abstract:

The Tat protein is able to translocate through the plasma membrane and when it is fused with other peptides may act as a protein transduction system. This ability appears particularly interesting to induce tissue-specific differentiation when the Tat protein is associated to transcription factors. In the present work, the potential of the complex Tat-MyoD in inducing equine peripheral blood mesenchymal stem cells (PB-MSCs) towards the myogenic fate, was evaluated. Results showed that the internalization process of Tat-MyoD happens only in serum free conditions and that the nuclear localization of the fused complex is observed after 15 hours of incubation. However, the supplement of Tat-MyoD only was not sufficient to induce myogenesis and, therefore, in order to achieve the myogenic differentiation of PB-MSCs, conditioned medium was added. The latter was obtained coculturing PB-MSCs with C2C12 without direct contact. These results suggest that TAT- transduction of Tat-MyoD, when supported by conditioned medium, represents a useful methodology to induce myoblasts differentiation.

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Abstract

The Tat protein is able to translocate through the plasma membrane and when it is fused with other peptides may act as a protein transduction system. This ability appears particularly interesting to induce tissue-specific differentiation when the Tat protein is associated to transcription factors. In the present work, the potential of the complex Tat-MyoD in inducing equine peripheral blood mesenchymal stem cells (PB-MSCs) towards the myogenic fate, was evaluated. Results showed that the internalization process of Tat-MyoD happens only in serum free conditions and that the nuclear localization of the fused complex is observed after 15 hours of incubation. However, the supplement of Tat-MyoD only was not sufficient to induce myogenesis and, therefore, in order to achieve the myogenic differentiation of PB-MSCs, conditioned medium was added. The latter was obtained coculturing PB-MSCs with C2C12 without direct contact. These results suggest that TAT-transduction of Tat-MyoD, when supported by conditioned medium, represents a useful methodology to induce myoblasts differentiation.

KEYWORDS: Tat-MyoD, equine PB-MSCs, C2C12, coculture, myogenic induction.

1. Introduction

Adult skeletal muscle presents a low cellular turnover in the absence of disease or damages (Cheung et l 2013). On the contrary, during regenerative mechanisms the muscle tissue becomes very dynamic thanks to the involvement of satellite cells. The use of these cells for therapeutic purpose appears promising for treatment of diseases and injuries affecting skeletal muscle, including muscular dystrophy (Partridge 2003). Both skeletal muscle injuries and disorders are actually quite common among athletic animals such as horses (Freestone and Carlson, 1991; Lee et al., 2016). However, the self-renewal potential of adult satellite cells is per se limited, decreases with age, sarcopenia (Chen and Goldhamer 2003) and is depleted by wasting muscular dystrophies (Yusuf and Brand-Saberi 2012). Given the need to use an unlimited cell population, mesenchymal stem cell (MSCs) deserves a particular attention to offer an alternative therapeutic solution for muscle diseases (Mizuno 2010). MSCs can be isolated from various anatomical districts such as bone marrow, adipose tissue, amniotic fluid, peripheral blood (Kuznetsov et al. 2001; Kern et al. 2006; Koerner et al. 2006; Martinello et al. 2010; Martinello et al. 2011) and they share the ability to differentiate along several pathways (Chamberlain et al. 2007; Giovannini et al. 2008). Up to now, scarce data are present in literature about the differentiation of MSCs into myoblasts. *In vitro*, it has been shown that MSCs may differentiate into skeletal muscle cells with conditioned medium as well as in coculture with a fusion between MSCs and myoblasts (Dezawa et al. 2005; Sung et al. 2013; Dugan et al. 2014). Specific signaling molecules, such as dexamethasone together with insulin and EGF (epidermal growth factor) (Tehrani et al. 2014), are able to induce the differentiation into skeletal muscle. Furthermore, MSCs isolated from bone marrow and treated with FGF (Fibroblast Growth Factors), forskolin, PDGF (Platelet-Derived Growth Factor) and transfected with an NICD plasmid were able to express MyoD (Dezawa et al. 2005), although the frequency of spontaneous cell fusion was very low. Recently, Rabiee et al. demonstrated that the overexpression of FND5, using an inducible lentivirus system, increased the transcription level for cardiac progenitors in embryonic stem cells (Rabiee et al. 2014) and Sung et al. induced equine MyoD expression in equine adipose-derived mesenchymal stem cell using a MyoD lentiviral vector

(Sung et al. 2016). Moreover, embryonic stem cells were induced to differentiate also into smooth muscle cells if Olfm2 (olfactomedin 2) overexpression was promoted (Shi et al. 2014). In a coculture of stem cells from amniotic fluid and cardiac cells, the physical contact between the two types of cells seems to be necessary but not sufficient to induce the cardiogenic potential (Gao et al. 2014); this fact means that a specific microenvironment is required to induce the maturation of myogenic cells. Therefore, the innovative approach of protein transduction with Tat domain fused with various transcription factors (Lin and Kao 2015; Woo et al. 2015), including MyoD (Sung et al. 2013; Hidema et al. 2014), appears to be a valid technical approach. Even though some data indicate that Tat-MyoD induces myogenic differentiation in naturally predisposed cells only, like the C2C12 cell line (Noda et al. 2009) or the mouse muscle primary cells (Hidema et al. 2014) Sung et al. demonstrated that myogenic differentiation of human adipose-derived stem cells was reached usingTat-MyoD transduction when the cells were fused with C2C12 myoblasts (Sung et al. 2013). In the present study, we described that myogenic differentiation of equine peripheral blood mesenchymal stem cells (PB-MSCs) using the Tat-MyoD transduction can be achieved simply with a coculture C2C12 myoblasts. .

2. Materials and methods

2.1.Generation of Tat-MyoD fused proteins

The nucleotide sequence encoding human MyoD was amplified from a human cDNA library with the following oligonucleotides (CAGCTAGCATGTCCTTCGCCATGCTGCGTTCAG - TGCAAGCTTCTAACTTCGAATCGCCGTCTTTTC) and cloned in plasmid Tat-Prp (Vicario et al. 2014) between *NheI* and *HindIII* restriction site, in order to obtain plasmid pTat-MyoD. The plasmid pTAT-MyoD is able to coding for MyoD sequence fused to peptide containing the translocation of HIV-1 protein TAT with 6x Histidine tag at N-terminus.

(NH_2-MRGSHHHHHHGMARGYGRKKGRQRRR-).

The plasmid pTat-MyoD was trasformed in Escherichia Coli BL21 bacteria cells. The bacteria were grown at 37°C in Luria Broth (LB) medium containing ampicillin (100 µg/ml) to an OD600 of 600 nm. Protein expression was induced by adding IPTG (Isopropil-β-D-1-Thiogalactopyranoside) about 4 hours at 25°C. To collect the Tat-MyoD protein, bacteria were harvested and cell membrane was lysed by sonication under denaturing condition using 6 M guanidinium. The proteins were bound to the resin IMAC and then were eluted with 8 M urea and 300 mM imidazole (pH 6.3). The fractions containing the larger quantity of protein were purified using a gel filtration PD10 column (GE Healthcare) to eliminate urea and imidazole. The purified protein was quantified using a spectrophotometer and then an SDS-PAGE was made to verify the purity of Tat-MyoD (44 KDa). The final protein concentration obtained was 0,5 mg/ml.

2.2.Transduction of Tat-MyoD into peripheral blood derived-mesenchymal stem cells (PB-MSCs)

MSCs were isolated from equine peripheral blood (Martinello et al. 2010) and were cultured in GM (growth medium, DMEM Dulbecco's Modified Eagle's Medium, 10% fetal bovine serum FBS, and antibiotics 100 mg/ml streptomycin, 100 U/ml penicillin, Euroclone) at 37°C. In order to evaluate the internalization of Tat-MyoD, PB-MSCs (when reaching confluence) were incubated in the presence of 0,1µg/ml Tat-MyoD for 2, 6, 15, 24 and 48 hours in medium without serum. The time course analysis was repeated in quadruplicate.

2.3.Coculture of PB-MSCs and C2C12

PB-MSCs and C2C12 cells were cocultered independently by using transwell insert (BD Falcon) with a 1µm pore size of membrane to separate each cell type. PB-MSCs were plated at the bottom

of 6-well plates at concentration of 1,5x10^5 cells/well in GM and the day after the cells were treated with Tat-MyoD for 15h in medium without serum. Concurrently, C2C12 were seeded at density of 3x10^5 cells per insert in GM, when the cells reached 80% of confluence the medium was changed to DM (differentiation medium, DMEM, horse serum 2%, antibiotics 1%, Euroclone). After 3 days the inserts with C2C12 were transferred into the wells with PB-MSCs in DM. The coculture was maintained for 7 days in DM and the experiment was repeated in triplicate.

2.4.Immunostaining

To perform immunostaining experiments cells were washed with PBS and fixed in 4% paraformaldehyde for 10 min; after further washing they were permeabilized with 0,3% Triton X-100 for 5 min and blocked for 1h using 1% FBS. Anti-His tag antibody (1:100, Sigma) was employed to evaluate the internalization of Tat-MyoD. To evaluate the differentiation of cells, anti-MyoD (1:100, Santa Cruz), anti-Myf5 (1:100, Santa Cruz) and anti-Myogenin antibodies (1:500, Chemicon) were used. All antibodies were maintained overnight at 4°C. Fixed cells were washed with PBS followed by addition of anti-mouse or anti-rabbit Alexa 568 conjugated antibody (Molecular Probes) at a 1:500 (v/v) dilution. Finally, staining of nuclei was obtained with DAPI (Sigma). As controls, PB-MSCs treated with Tat-MyoD without coculture and PB-MSCs in coculture, but without Tat-MyoD treatment, were used.

3. Results

3.1.Purification of Tat-MyoD protein

Tat-MyoD was expressed in *E. Coli* Bl21 and purification was performed using a Ni-NTA column. Tat-MyoD purified to homogeneity shows and apparent molecular weight of 44 KDa on SDS-

PAGE and migrate on gel slower respect its theoretical molecular weight of 37905.1 Da (Fig. 1). This common behavior may be explained due to the high number of basic amino acids (17.2% respect to total amino acids).

3.2.Localization of Tat-MyoD into PB-MSCs

In order to evaluate the cellular pathway of Tat-MyoD protein construct, an immunofluorescence assay was chosen (Fig. 2). Using confocal microscopy, it was found that after 2 and 6 hours of PB-MSCs treatment with MyoD-Tat, the protein permeated cell membrane and was present in the cytoplasm; only after 15 hours of incubation, the construct was confined in the nucleus and this localization was persistent after 24 and 48 hours of treatment (Fig. 2). Experiments were performed in serum free medium since the latter inhibits this process (data not shown).

3.3.Myogenic differentiation of PB-MSCs

Myogenic differentiation was achieved using Tat-MyoD transduction and the inductive medium of the cellular line C2C12. To study the effect of our set up on myogenic marker expression in PB-MSCs, we performed an indirect coculture using transwell insert (Fig. 3B). The scheme of experiment is illustrated in Figure 3A. The effective differentiation was evaluated observing the localization of Myf5 and Myogenin by immunofluorescence (Fig. 4). Results indicated that to activate the myogenic pathway in mesenchymal stem cells it was necessary the co-action of MyoD transduction and the molecular signals present in the medium of C2C12. Figure 4 (A, B) shows Myf5 and Myogenin expression in PB-MSCs treated for 15 hours with Tat-MyoD in serum free medium and, subsequently grown for 7 days in coculture with C2C12 myotubes in differentiative medium. The myogenic differentiation of PB-MSCs was not achieved using, separately, Tat-MyoD

(Fig. 4 D, E) or the C2C12 conditioned medium (Fig. 4 G, H). Fig 4C and 4F show the internal localization of Tat-MyoD complex by means of His-Tag antibody and fig. 4I confirms the absence of myogenic differentiation with only C2C12 conditioned medium with the use of MyoD antibody.

4. Discussion

The equine model offers a unique opportunity to explore treatment strategies for musculoskeletal disorders under conditions similar to the pathophysiology of human patients. Current treatments are often restricted to the management of symptoms or replacement with inert materials; therefore, there is a need for alternative biological approaches. MSCs may differentiate into cell types relevant to amend musculoskeletal diseases (Gupta et al. 2007; Lee et al. 2011; Galli et al. 2014) and are able to secrete growth factors to promote a repairing environment. However, for cell therapy purposes is necessary that MSCs are able to participate in the formation of new muscle fibers, a critical process that has not been fully elucidated so far. In vitro, hASCs (Human adipose-derived stem cells) treated with 5-azacytidine and fibroblast growth factor-2 (FGF-2) stimulates the early muscle differentiation steps (Eom et al. 2011); more, the expression of MyoD using high efficient lentiviral transduction induces myogenic differentiation while adipogenic differentiation is inhibited (Goudenege et al. 2009). Moreover, using MyoD lentiviral vector Sung et al. induced the expression of MyoD but not of Myogenin, (Sung et al. 2016). However, these methods are not appropriated for clinical use due to their mutagenic potential. In the last decade, several groups have demonstrated that the Tat protein transduction domain (PTD) is a great transactivator of gene expression (Dietz and Bähr 2004; Fittipaldi and Giacca 2005); its short amino acid motif, highly enriched in basic amino acids, binds to the cell surface and internalize in a variety of different cell types. In the recent past, various cellular proteins were described to interact with Tat and mediate or control its transcriptional activity (Kashanchi et al. 1996; Benkirane et al. 1998; Marzio et al. 1998; Col et al. 2001). In the present study, the human MyoD protein was engineered with the Tat sequence in order

to evaluate a safe method for the induction of mesenchymal stem cells towards the myogenic differentiation. This approach was already proposed in cells that naturally follow the myogenic fate, as mouse myogenic primary cells (Noda et al. 2009) and C2C12 cell line (Hidema et al. 2012) but to our knowledge was never tried on PB-MSCs. Additionally, experiments from Sung et al. (2013) underlines the importance of the extracellular environment, as they were able to differentiated human adipose-derived stem cells into myogenic cells using a fusion with C2C12 cells.

We were successful in inducing myoblasts differentiation in PB-MSCs. Our experiment indicates that the development of myogenic phenotypes of mesenchymal stem cells by Tat-MyoD construct depends on time and culture conditions, highlighting the role of *in vitro* microenvironment in terms of secreted factors and cell contacts.

Indeed, an important observation raised from our experiments was the necessity to add Tat-MyoD in a cell culture with serum free medium. It has been demonstrated that short peptides (Green and Loewenstein 1988) rich in arginine (Suzuki et al. 2002) are rapidly internalized by cells, in a receptor-independent manner and without energy consumption. This does not happen for Tat basic domain when fused to protein cargos (Fittipaldi and Giacca 2005). It was suggested that the process of Tat internalization occurs through adsorptive endocytosis. Several investigators (Hakansson et al. 2001; Mann and Frankel 1991) state that Tat sequence binds homologue of heparin sulfate (HS) glycosaminoglycan (GAG), a major constituent of extracellular matrix, suggesting that the bound HS/Tat might be involved in the internalization process. In accordance with this hypothesis, our study suggests that the presence of heparin in serum competes with the bound of HS/Tat, decreasing the uptake progression. To stimulate myogenic differentiation, Tat-MyoD has to be localized in the nucleus. Our results demonstrated that after 2 and 6 hours the construct remained in the cytoplasm, probably in vesicle as hypothesized by (Noda et al. 2009). Only after 15 hrs of incubation, Tat-MyoD was localized in the nucleus where it persisted after 24 and 48 hrs. However, the activation of myogenic pathway by nuclear MyoD was not sufficient to induce cellular differentiation..

Likewise PB-MSCs cocultured with C2C12 grown in cell insert (prevent the cell direct contact but permits the interaction of culture medium) was not enough to induce the myogenic commitment.

To our knowledge, this is the first study that shows a myogenic differentiation in equine adult stem cells using the TAT-mediated protein transduction system; the advantage of our method consists in obtaining committed myogenic cells derived from an abundant cell source, as PB-MSCs, without the need of fusion with other cells. It is important to state that our model might easily be reproduced also in human mesenchymal stem cells too (Martinello et al, unpublished results) although further studies will be necessary to develop this methodology for clinical purposes.

Acknowledgments

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Conflict of Interest: None

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Figure Legends

Fig. 1. Purification of Tat-MyoD by Ni-NTA column. Lane 1, BL21 cell and Sumo-hyrudin. Lane 2, BL21 and pTat-MyoD before induction. Lane 3, BL21 and pTat-MyoD after induction with 0.5 mM IPTG. Lane 4, Purified Tat-MyoD after Ni-NTA column.

Fig. 2. Immunofluorescence analysis of PB-MSCs treated with Tat-MyoD for 2, 6, 15, 24 and 48 hours using the anti-His Tag antibody (red) and DAPI (blue). From 15 hours of incubation anti-His Tag and DAPI colocalized. Bottom right image shows PB-MSCs after 48 hours of Tat-MyoD incubation (PC = Phase contrast). Scale bars: 58µm

Fig. 3. (A) Scheme of coculture between PB-MSCs treated with Tat-MyoD and C2C12, GM indicates growth medium and DM differentiation medium. (B) Scheme of transwell insert used for the coculture.

Fig. 4. Myogenic differentiation of PB-MSCs. Immunofluorescence of PB-MSCs after the Tat-MyoD treatment and the contemporary coculture with differentiated C2C12 (A, B, C). Immunofluorescence of PB-MSCs after 7 days of Tat-MyoD treatment (D, E, F) and after 7 days of coculture with differentiated C2C12 (G, H, I). The images show the merge between nuclear DAPI

staining (blue) and anti-Myf5 (A, D, G), anti-Myogenin (B, E, H), anti-His Tag (C, F), and anti MyoD (I) antibodies (red staining). Scale bars: 58 µm.

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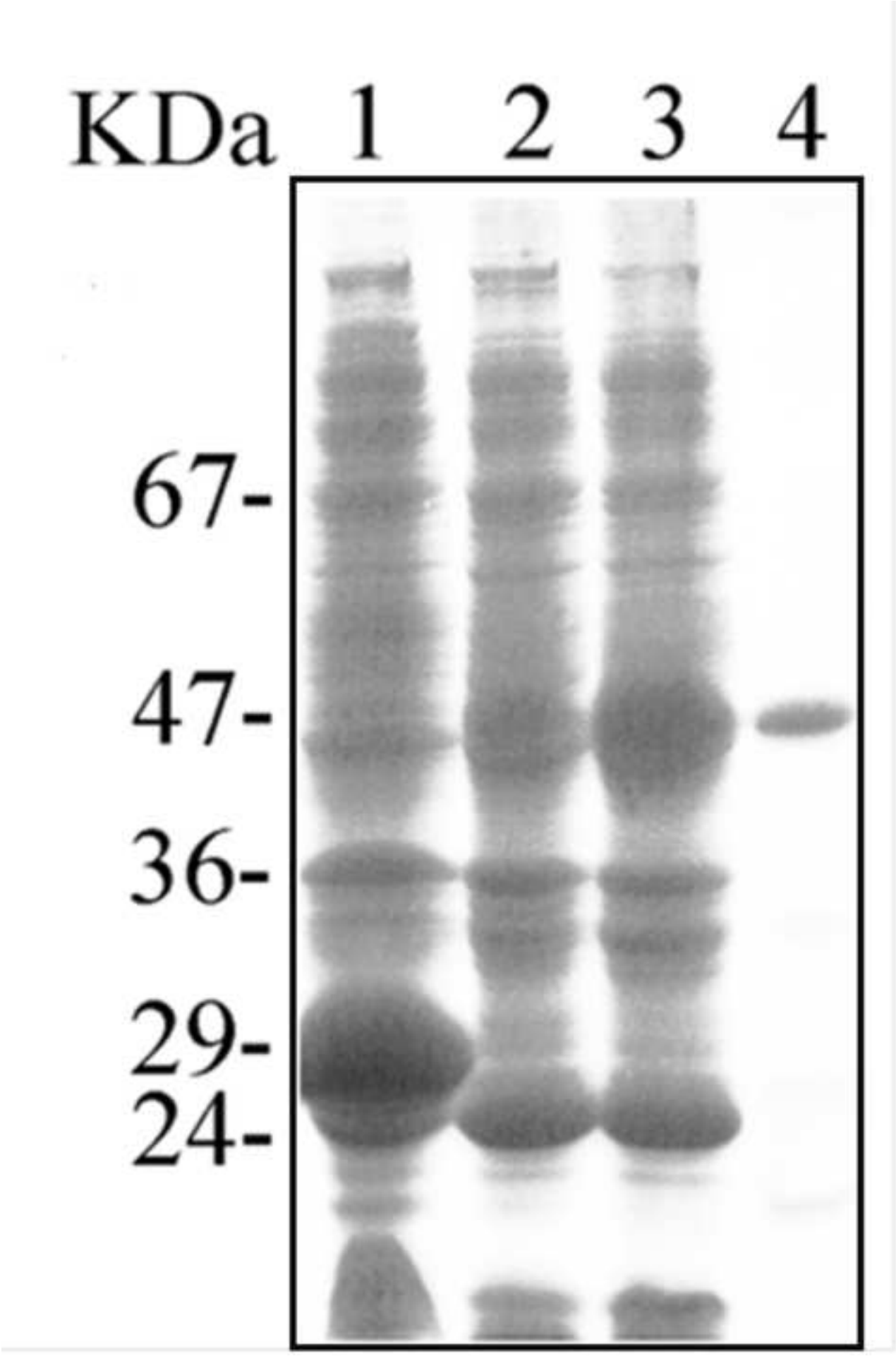

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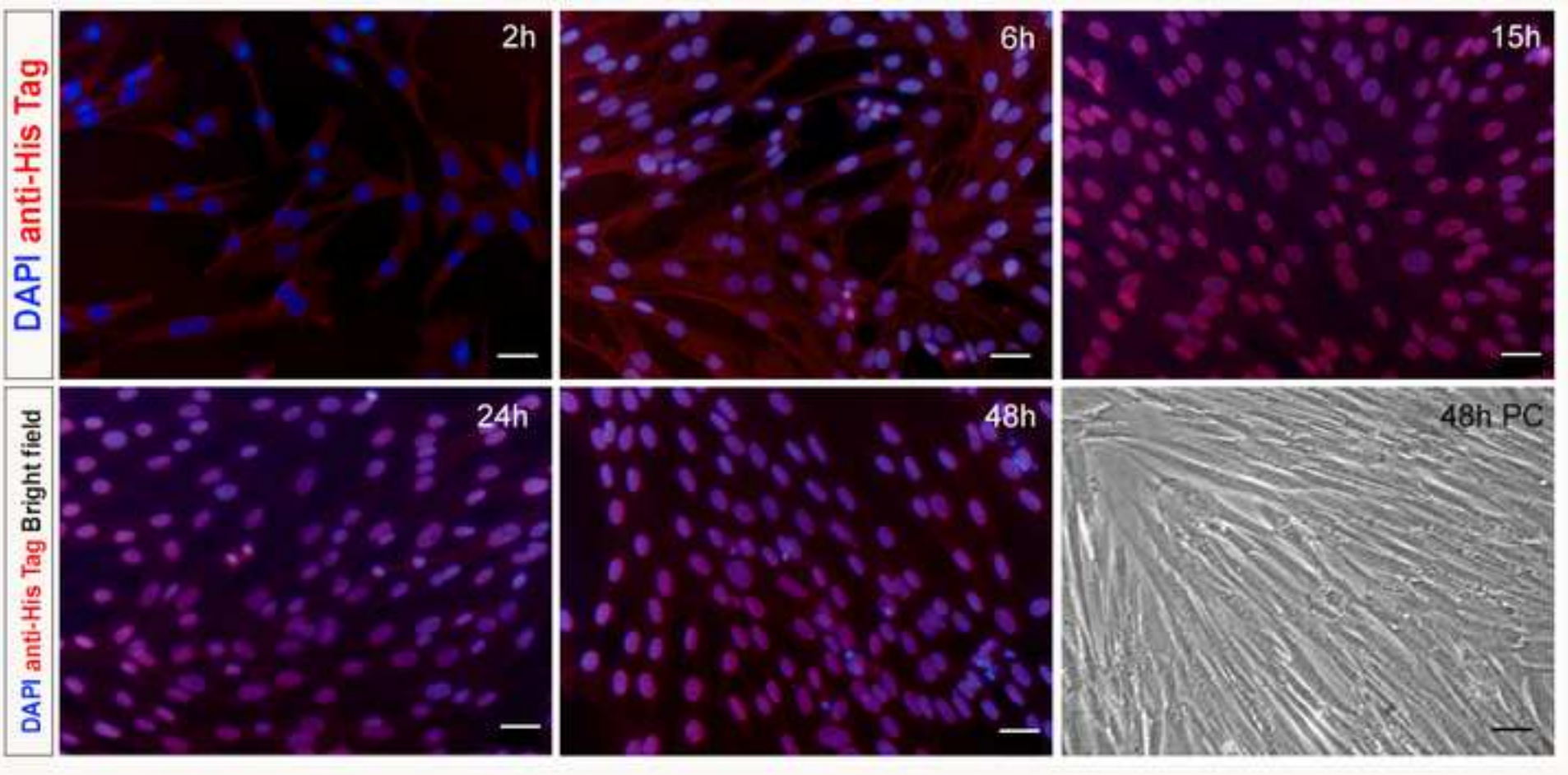

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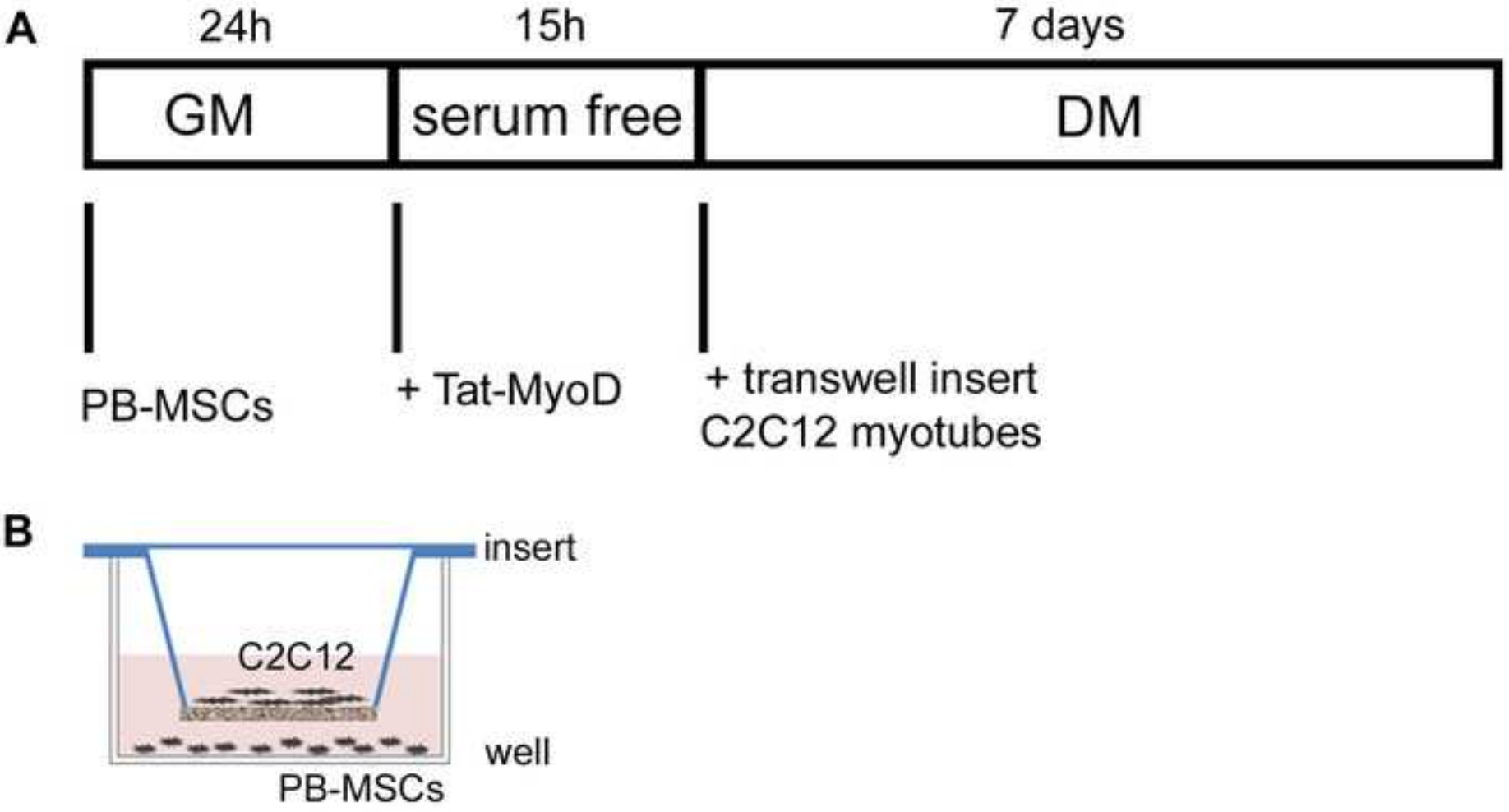

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