Editorial

Making muscles from stem cells—miRNAs enter the stage

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Pluripotent stem cells (PSCs), such as induced pluripotent stem cells (iPSCs), are able to differentiate into any given tissue building mammalian organism. For this reason, they are considered as a universal source of cells to treat various conditions and diseases. They are also proposed as the ones that could be used to treat dysfunctional muscles, such as in Duchenne's muscular dystrophy (DMD) patients. In case of DMD lack of dystrophin causes skeletal muscle, damage followed by regeneration, which relies on the presence of muscle stem cells, i.e., satellite cells (SCs). SCs, in response to the muscle injury, activate, differentiate into myoblasts, fuse and reconstruct multinucleated myotubes and myofibers. Repeated cycles of injury and repair, characteristic for dystrophic muscles, lead to the depletion of SC population limiting muscle regenerative potential. Thus, in case of such condition affecting muscle function transplantation of properly selected cells, i.e., the ones that reveal myogenic potential, might be therapy of choice. However, to make such therapeutic approach possible molecular basis of PSC differentiation need to be fully understood and differentiation methods perfected and readily available.

Research projects focusing at the myogenic differentiation of PSCs and scheming differentiation protocols take advantage either of overexpression of selected genes or base on special culture conditions facilitating myogenic differentiation. Among crucial regulators of skeletal muscle development are Pax3 or Pax7 transcription factors. Their overexpression in PSCs or cells derived from them, followed either by cell sorting or 3D culture, as recently showed by Rao *et al.*, allowed efficient turning of PSCs, or cells derived from them, into myogenic precursor cells [e.g., (1-6)]. Also, non-invasive methods, such as supplementing culture media with growth factors or cytokines, were shown to facilitate and improve myogenic differentiation of PSCs [summarized in (7,8)]. Within last several years protocols relying on the mouse or human PSC culture in the precisely adjusted and temporally changing environment, mimicking embryonic development, was published [e.g., (9-11). Scheme proposed by Chal *et al.* included sequential delivery or manipulation of factors expressed and active at subsequent stages of mouse embryonic development. Such approach allowed mesoderm induction and generation of myoblasts able to participate in skeletal muscle regeneration (9,10,12,13). In the meantime, other research groups, including our own (14), tested the possibility of application of molecules, such as microRNA (miRNAs) to control PSC differentiation. Many of presented studies included the detailed transcriptome analysis in PSCs grown in colonies (15) and also differentiating in spherical structures, so called embryoid bodies (EBs) and EB outgrowths (EBOs) (14,16,17). Among the research groups which are testing myogenic potential of various stem cell, including PSCs, and also working to design protocols of their differentiation is the one led by Maurilio Sampaolesi [e.g., (6,18-21)].

In 2017 Sampaolesi team published the study which was a continuation of the previous experiments resulting in generation of mesodermal progenitor cells from iPSCs derived from somatic cells of such species as mouse, rat, or dog (6). This time the Authors focused at human cells asking if mesodermal, iPSCs-derived progenitors (MiPs) could be generated with the help of methods previously proposed by Quattrocelli *et al.* who differentiated animal

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iPSCs within EBs, sorted CD140a+/CD140b+/CD44+ cells, and also analyzed the fate of resulting cells after Pax3/Pax7 overexpression, co-culture with C2C12 myoblasts, or differentiating within cardiac or skeletal muscles (6). Current investigation validated the previously proposed MiPs derivation protocol as the one applicable for human cells. As it was done in the past, MiPs were generated from different cell lines, i.e., from fibroblasts and mesoangioblasts (MAB)-derived iPSCs (22). Comparison of these cell lines revealed that, as described by others, source of cells from which iPSCs were generated does matter (23). As shown by Giacomazzi *et al.* "origin" of the iPSCs impacted the pattern of variety of genes expressed in MiPs and also the regenerative capacity of these cells (22). All generated cells were tested *in vitro* and in *in vivo* models, i.e., transplanted intravenously or into damaged muscles. Interestingly, MiPs originating from human mesoangioblast-derived iPSCs were characterized by better ability to improve skeletal muscle regeneration than those one obtained from fibroblasts. Such differences were not manifested once the cells were used to treat cardiac muscles. Thus, it was proven that MiPs-derived cells are able to improve skeletal muscle regeneration and also cardiac muscle function. Detailed transcriptome analyzes showed that both types of MiPs expressed mesodermal and myogenic markers, such as PAX3, MEF2C, MYOM2/3 and others. Importantly, AGRIN and UTROPHIN were upregulated in these cells. As mentioned above, this analysis revealed the differences depending of the MiPs origin—for example several myogenic inhibitors were upregulated in fibroblast-derived MiPs and the level of several myogenesis-associated transcripts was higher in MAB-MiPs, suggesting that the choice of cells used for iPSCs generation could be of great importance. The Authors also focused at the epigenetic status of analyzed cells.

Results of transcriptome analyzes allowed to define anti- and pro-myogenic factor pools in tested cells which than were validated *in vitro* to prove that anti-myogenic pool genes, such as *LHX2*, *LTBP4*, *MYB*, *OSTN*, *SMAD5* were downregulated in the reaction of gene-targeting, pro-myogenic cocktail. In contrary, the expression pro-myogenic genes *ANXA3*, *ANXA7*, *PAX7* and *SMAD7* was decreased in the reaction to anti-myogenic cocktail. Further *in vitro* experiments have proven that abovementioned cocktails either increased or decreased, as expected, the ability of MiPs to fuse with C2C12 myoblasts and to form hybrid myotubes.

The major novelty of the study relies, however, on the modulation of miRNA levels as a tool to improve myogenic differentiation of stem cells. Our own group has previously shown, that the transient increase in the level of properly chosen, single miRNA can change the fate of differentiating PSCs. For example, by expressing miR181 in mouse ESCs we were able to increase the number of myogenic cells forming multinucleated myotubes (14). Giacomazzi *et al.* proposed cocktails of several molecules modulating myogenic ability of MiPs, manifested for example by the size of myotubes formed in co-culture of MiPs with C2C12 myoblasts (22). It would be, however, interesting to evaluate the impact of tested cocktails on differentiation of MiPs cultured without myoblast "support". Presented approach resulted from detailed analysis of miRNAome of MiPs originating from fibroblast- or MAB-derived iPSCs. Such investigation allowed to identify miRNAs expressed in MiPs and associated with mesodermal progression and myogenesis, e.g., let7a, miR1, miR590, miR497, miR34a, miR27b, miR101, miR133a, miR138, and other. Interestingly, as shown by previous tests, differences between MiPs originating from fibroblast- and MAB-derived iPSCs were also pretty apparent. Next, on the basis of these analyzes another sets of miRNA inhibitors and miRNA mimics were selected, including pro-myogenic and anti-myogenic miRNA, i.e., experimental scheme "perturbing" the levels of selected miRNAs was designed. Such cocktails were then again validated by *in vitro* co-culture of treated MiPs with C2C12 myoblasts what allowed to precisely follow their ability to fuse and form hybrid myotubes with myoblasts. The pre-treated cells were either intravenously injected or transplanted into the cardiac muscles of immunodeficient mice. Thus, quality of proposed miRNA modulating cocktails were assessed proving the quality of those ones that aimed to improve and those one that was expected to block myogenic differentiation. The Authors documented that miRNAs are indeed potent regulators of cellular processes that can be used to improve cell-based therapies. But, are the proposed methods of myogenic cells generation feasible to be used in the future in the clinics?

Methods, such as proposed by Borchin *et al.* (11) and Chal *et al.* (9) base on the precisely controlled culture conditions; Bem *et al.* (14) propose transient overexpression of miRNAs—thus, these methods do not involve genetic manipulations of the stem cells and for this reason could be considered safe. Importantly, at least some of them yielded high proportion of myogenic cells [e.g., (9)]. Giacomazzi *et al.*, presented approach which also resulted in high

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frequency of myogenic cells and significant improvement of cardiac function and skeletal muscle regeneration. However, as in case of other protocols, this multi-step schemes might be difficult to be routinely repeated. It covers derivation of iPSCs, their *in vitro* differentiation, sorting and processing of MiPs, transient treatment with cocktails including miRNAs and miRNA inhibitors (22). Still, this study shows how to select such factors as miRNAs to manipulate stem cell differentiation, and how to pinpoint the molecules which play important role in the myogenic differentiation and could be used in the future to efficiently derive cells of therapeutic value. It also presents future directions for creating simplified protocols allowing to derive myogenic cells. Rapidly developing miRNA field shows that these molecules are more and more frequently used to control the differentiation of stem cells. Hopefully, in the future, generation of human myogenic progenitors will be done using the protocols taking advantage of non-invasive methods relying for example on modulating the levels of miRNAs or precisely designed culture conditions.

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Footnote

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