Author's response to reviews

Title: Overexpression of connexin 43 using a retroviral vector improves electrical coupling of skeletal myoblasts with cardiac myocytes in vitro

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Author's response to reviews: see over
To: BMC Cardiovascular Disorders

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Dear Editor,

We are grateful for all the extensions granted to us for the revision of our manuscript “Overexpression of connexin 43 using a retroviral vector improves electrical coupling of skeletal myoblasts with cardiac myocytes in vitro”. We now submit our revision of the article.

We respond below to the reviewers comments (their text is in blue) and add a paragraph to the Discussion section prompted by the reviewer 2.

Referee 1.

General

In this study, the authors generated an efficient bicistronic retroviral vector (MLV-CX43-EGFP) for the overexpression of connexin 43 in skeletal myoblasts. A more than four-fold overexpression of connexin 43 in the transduced skeletal myoblasts, is shown. Functionality of the overexpressed connexin 43 is demonstrated by microinjection of a fluorescent dye showing enhanced gap-junctional intercellular transfer in connexin 43 transduced myoblasts. Cocultures of rat cardiac myocytes with connexin 43/EGFP transduced skeletal myoblasts in multielectrode array culture dishes show elevated extracellular field action potential activation rates, similar to the rates in pure cultures of cardiac myocytes. The authors conclude that retroviral connexin 43 transduction may be employed to augment engineering of the electrocompetent cardiac grafts from patients’ own skeletal myoblasts.

Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached).

Technically, this a good study but unfortunately, it also has a major flaw. If one proposes to use skeletal myoblasts for cardiac repair, then one should anticipate that these cells do not remain undifferentiated once implanted in the heart. Instead,
skeletal muscle biology and multiple previous grafting studies teach us that these cells will undergo fusion and differentiation, thus giving rise to mature skeletal myofibers. Exactly this feature is the very reason skeletal muscle cells are used for this purpose; the hope that these cells mature and support cardiac contractility actively by coupling to host myocardium electromechanically. Now, the authors here present convincing evidence that their connexin43 transfected skeletal myoblasts can do the trick, i.e. couple to one another and to neonatal cardiomyocytes. We should also realize that even non-transfected myoblasts, in principle, can do this because they express the necessary gap junction and adherens junction molecules (connexin43, N-cadherin). The big question is: What will happen if they study differentiated myotubes instead of myoblasts? In skeletal muscle development endogenous connexin43 and N-cadherin are shut down as the skeletal muscle fiber matures. The authors need to show that expression from the retroviral cassette persists after differentiation into myotubes. Likewise, they need to show that these myotubes can sustain coupling to cardiac myocytes. Once we know the answers to these questions, we can feel much more comfortable to remotely think about clinical applications.

In the manuscript we say that a tandem of MLV and CMV promoters is likely to ensure absence of connexin 43 expression shutdown after differentiation of myoblasts into myotubes. Indeed, a tandem arrangement of two promoters is a reflection of our desire to guarantee expression post-differentiation as much as possible. It is very difficult to prove our assertion experimentally. Following a number of published reports, we extensively studied induction of myoblast differentiation \textit{in vitro} by horse serum. We saw induction of limited cell fusion involving up to 5\% of the cells, but no total conversion to myotubes. Because of the background of non-differentiated myoblasts we had difficulty in determination of connexin 43 expression level after differentiation. We envisage even greater technical difficulties in addressing this question \textit{in vivo}, this study would be clearly out of scope of the manuscript.

Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

Why was the human connexin43 gene chosen (and not the rat)?

Our choice of human connexin 43 cDNA as a transgene was dictated by our desire to distinguish between expression of endogenous connexin 43 and expression of transgenic connexin 43 by restriction analysis of connexin 43 RT-PCR amplicons. We obtained these data which clearly showed concomitant expression of rat (endogeneous) and human (transgenic) connexin 43 in transduced myoblasts. We can add the RT-PCR data to the manuscript, if requested.
Referee 2.

General:
In this well designed, clearly written manuscript the authors provide useful indications on the best procedure to achieve autologous skeletal myoblasts genetically engineered for stable over-expression of the gap junction protein Cx43. The authors also provide evidence that Cx43-transduced myoblasts exhibit higher efficiency of gap junctional coupling: in fact, vital dye spreading between adjacent myoblasts is enhanced as compared with wild-type cells, and propagation of electrical waves corresponding to action potentials from cardiomyocytes to myoblasts in mixed cultures is also increased. These findings fit well with our observations, reported in a recent article (Formigli L et al., Am J Physiol-Cell Physiol 288; C795-C804, 2005), in which we induced the overexpression of Cx43 by C2C12 myoblasts treating them with the hormone relaxin, and we observed increased intercellular coupling among myoblasts and between myoblasts and cardiomyocytes.

I have been impressed by the surprisingly high yield of engineered myoblasts that can be obtained by the described method, especially considering that these cells are freshly isolated cells and not a stabilised cell line. Nonetheless, it appears that these myoblasts have been successfully cultures up to 12 weeks. Can the authors rule out that genetic manipulation may have altered the cells' growth control mechanisms, or may have rendered them insensitive to proapoptotic stimuli? I am aware that this is not a crucial issue to the present in vitro study, but may become of extreme importance in view of a possible clinical application of genetically engineered myoblasts in post-infarcted patients, because of their putative increased tumorigenic potential. The authors may wish to discuss this issue.

Our observations show that confluent cultures of primary myoblasts can stay alive for at least a month in the medium supplemented with fetal calf serum (and considerably longer in a medium without serum). This property of primary myoblasts remained unchanged during all the passages we made and was in stark contrast to the transformed rat L6 myoblasts, which could die in a week after achieving confluency. Thus, fortuitously, the number of passages (and, therefore, cell divisions) required for our manipulations of primarily myoblasts (magnetic sorting, retroviral transduction, preparative FACS) was lower, than a number of passages for analogous manipulations with a permanent myoblast cell line L6.

We add the above paragraph to our Discussion section.

Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached):
I feel the need for additional dye transfer experiments on myoblasts and cardiomyocytes in co-culture, aimed at evaluating - and possibly quantifying – the extent at which Cx43 overexpression by myoblasts actually potentiates gap junctional coupling between the two different cell types. Such experiment should be pretty simple to the authors.
We used the extension granted by BMC Cardiovascular Disorders to perform dye transfer experiments in co-cultures of cardiac myocytes with connexin 43 transduced skeletal myoblasts and non-transduced skeletal myoblasts. We were unable to show definitively what is happening in terms of dye transfer. The difficulty was in unexpected flattening of the cells in co-cultures, which complicated microinjections.

Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct):
None is needed. The manuscript is very well written and arranged.

Discretionary Revisions (which the author can choose to ignore):
None.

Referee 3

This is a well written study which needs little correction. However there are not many new findings from three studies that have already been published:

I would still highly recommend this study for publication because it is still of interest to the readers and would contribute significantly to our knowledge of genetic modification of myoblast. If the authors could include in vivo study data that would be more interesting: to evaluate the modified myoblasts for treating heart failure.

The matter under investigation is far from trivial and extremely challenging from the technical point of view. Our study adds new knowledge to the papers indicated above. Suzuki et al used transfection as a method of connexin 43 gene delivery followed by selection of drug resistant clones of permanently transformed L6 skeletal myoblasts (not primary skeletal myoblasts). This is hardly a realistic approach for human therapy. We believe that our state of the art retroviral technology (e.g. completely novel synergy between Transfectam and Retronectin enhancement of primary myoblast transduction) and original electrophysiological observations represent a new important advance compared to the studies of Reinecke et al and Abraham et al.

Truly yours,
Dr. Oleg Tolmachov