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Original Article

Tracking the Fate of Muscle-derived Stem Cells: an Insight into the Distribution and Mode of Action

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Purpose: To examine the fate of muscle-derived stem cells (MDSC) after injection into different host conditions and provide an insight for their mechanism of action.
Materials and Methods: MDSCs differentiated in vitro towards the endothelial lineage and transfected with lentivirus tagged with green fluorescent protein (GFP) were injected into two animal models mimicking vascular diseases: hindlimb ischemia and carotid injury models. Injected cells were tracked at the site of injection and in remote organs by harvesting the respective tissues at different time intervals and performing immunofluorescent histological analyses. Stem cell survival was quantified at the site of injection for up to 4 weeks.
Results: MDSCs were successfully tagged with fluorescent material GFP and showed successful implantation into the respective injection sites. These cells showed a higher affinity to implant in blood vessel walls as shown by double fluorescent co-stain with CD31. Quantification of stem cell survival showed a time-dependent decrease from day 3 to 4 weeks (survival rate normalized against day 3 was 72.0% at 1 week, 26.8% at 2 weeks and 2.4% at 4 weeks). Stem cells were also found in distant organs, especially the kidneys and liver, which survived up to 4 weeks.
Conclusion: MDSCs were successfully tracked in different vascular disease models, and their fate was assessed in terms of cell survival and distribution. Better understanding of the donor cell properties, including their interaction with the host conditions and their mechanism of action, are needed to enhance cell survival and achieve improved outcomes.

Key Words: Vascular diseases, Stem cell niche, Survival, Adult stem cells

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INTRODUCTION

Adult somatic stem cells are widely being studied due to their potential for use in regenerative medicine, and the recent amount of knowledge in this field has increased dramatically. The multipotency of these cells allow for differentiation into almost any desired lineage, and its self-renewal capacity ideally allows for unlimited reproduction of desired effects without the need to replenish them. Unfortunately, many studies have shown that stem cells injected into different tissues do not survive long enough to obtain prolonged effects [1-3]. Teratogenicity is also

another important issue with stem cell therapy, where stem cells differentiate in an uncontrolled fashion and into undesired lineages, although adult somatic stem cells are less prone to teratogenesis than pluripotent embryonic stem cells [4,5].

Recently the niche environment has been proposed to be a crucial factor in determining the behavior of stem cells. The niche can be described as a three dimensional microenvironment that regulates stem cell survival, maintenance, proliferation and differentiation through interactions with supporting niche cells, growth-modulating soluble factors and surrounding extracellular matrices [6]. Stem cell niches are likely to exist in all adult tissues, but only a few tissue-specific stem cell niches are known [7]. Niche conditions vary according to both the type of stem cells used and the tissue the stem cell is targeted, and previous studies have demonstrated the importance of the niche environment on stem cell fate [8,9]. The route of stem cell delivery is also another important determinant of stem cell action [10]. Therefore identification of relevant tissue-specific stem cell niches has been the focus of intense research.

In this study, muscle-derived stem cells (MDSC) were injected into two different vascular disease animal models: a hindlimb ischemia model (intramuscular delivery) and a carotid injury model (intravascular delivery). These cells were tracked longitudinally at the site of injection and in distant organs in an attempt to provide insights into the fate of injected stem cells in this specific stem cell population. The correlation between stem cell fate and previous observations on vascular disease improvement are also discussed.

MATERIALS AND METHODS

1) MDSC preparation

This study was approved by the Institutional Animal Care and Use Committee of Seoul National University Bundang Hospital (BA0712-018/025-01). Muscle-derived stem cell isolation, culture, differentiation and transduction with green fluorescence protein (GFP) marker were done using protocols previously described [11]. In brief, the gastrocnemius muscle from Sprague-Dawley (S-D) rats and Balb/c mice were harvested and MDSCs were isolated using the modified version of the preplate technique [12], which separates different populations of cells based on their adhesive properties on collagen-coated flasks. After 6 passages, the cells were further cultured for 1 week and CD34(+) cells were sorted with miniMACS (Miltenyi Biotec Inc., Cologne, Germany). In vitro differentiation of MDSCs to the endothelial lineage was performed using both shear stress and vascular endothelial growth factor (VEGF), and GFP marker was attached by lentiviral transfection of differentiated MDSCs with lenti-hCMV-GFP (Macrogen, Seoul, Korea).

2) MDSC injection into different animal models

Differentiated MDSCs tagged with GFP (MDSC-GFP) were injected into two different animal models mimicking vascular diseases. The first model consisted of a hindlimb ischemia model in Balb/c mice which was created by meticulous dissection and resection of 1 cm of the left femoral artery. The second model consisted of a carotid injury model in S-D rats which was created by meticulous exposure of the common, internal and external carotid arteries, after which endothelial denudation of the common carotid artery intima was performed by passing a 2 Fr balloon catheter back and forth for 3 times along a 1 cm length. In the hindlimb ischemia model, 1×10^7 cells of MDSC-GFP were injected intraoperatively and at day 2 in the hindlimb at 4 different locations (intramuscular delivery), while in the carotid injury model, 1×10^7 cells of MDSC-GFP were incubated in the lumen of the carotid artery for 30 min before restoration of blood flow (intravascular delivery). The same volume of saline was injected for the respective control groups.

3) Stem cell fate tracking

Animals were sacrificed for harvesting at different time points to track the fate of stem cells. In the hindlimb ischemia model, mice were sacrificed at day 3, 1 week, 2 weeks and 4 weeks and the hindlimb tissues were harvested for immunofluorescent staining. In the carotid injury model, rats were sacrificed at 1 hour, 1 week and 4 weeks and remote organs, including the heart, liver and kidneys were harvested to determine any distant migration of stem cells from the site of injection. Immunofluorescent staining was performed by immersion of harvested tissues in sucrose solution and fixation with tissue-TEK OCT compound (Sakura Finetek, Tokyo, Japan). For CD31 co-stain, the primary antibody CD31 (1:100; Abcam, Cambridge, UK) and the respective secondary antibody linked with rhodamine were sequentially added and cultured. The tissues were mounted using a mounting solution conjugated with 4',6'-diamidino-2-phenylindole (Vector Laboratories Inc., Burlingame, CA, USA).

Quantification of residual stem cell activity at different time points was done by counting the number of GFP positive cells from 4 random high-power microscope fields with Image Pro Plus 4.5 (Media Cybernetics, Warrendale,

PA, USA) and the mean value was calculated.

RESULTS

1) Lenti-hCMV-GFP uptake by differentiated MDSCs

MDSCs expressing characteristic features (positive expression of Sca-1 and CD34, indeterminate expression of desmin and no expression of CD45) were used in this study, and the endothelial differentiation process in vitro was in accordance with previous studies, showing positive expression of vascular endothelial cadherin, von Willebrand factor, Flk-1 and CD31 [11]. Transfection of these cells with lenti-hCMV-GFP showed a characteristic green color when the cultured cells were visualized in vitro under immunofluorescent staining (Fig. 1A).

2) MDSC-GFP implantation into ischemic hindlimbs

MDSC-GFP injected into ischemic hindlimbs of mice showed successful implantation at the site of injection at day 3, 1 week and 2 weeks when viewed under immunofluorescent staining (Fig. 1B). The cells were more prominently located in the intima layer of the muscle capillaries, which suggests a higher affinity of these endothelially differentiated cells to locate in blood vessels. When co-stained with CD31 (an endothelial marker), the implantation of MDSC-GFP in the intima layer was more evident, as shown by the merging of GFP (green color) and CD31 (red color) to show a characteristic yellow stain (Fig. 2). This demonstrates the affinity of MDSC-GFP to adhere to the endothelium of muscle capillaries, probably as part of the angiogenic process in response to the ischemic insult. However, there is no further expression of GFP at 4 weeks, as shown by the lack of green colored stain in

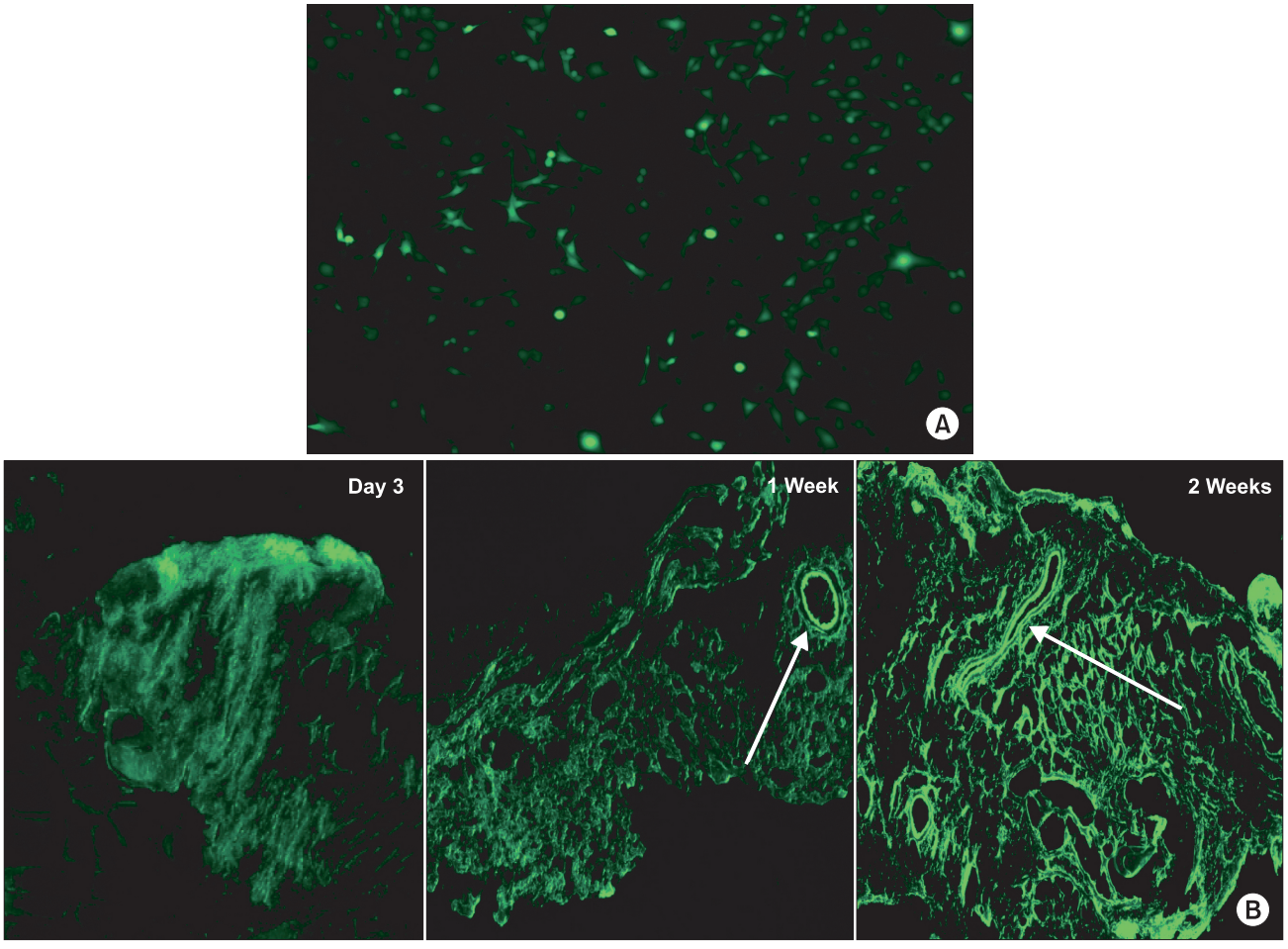

Fig. 1. Transfection of muscle-derived stem cells (MDSC) with green fluorescence protein (GFP) and injection into ischemic hindlimbs of Balb/c mice (x100). (A) Immunofluorescent cytological staining showing successful transfection of MDSCs with lenti-hCMV-GFP. (B) Successful implantation of MDSC-GFP after injection into ischemic hindlimbs, with higher affinity of cells to implant in muscle capillary vessels (arrows).

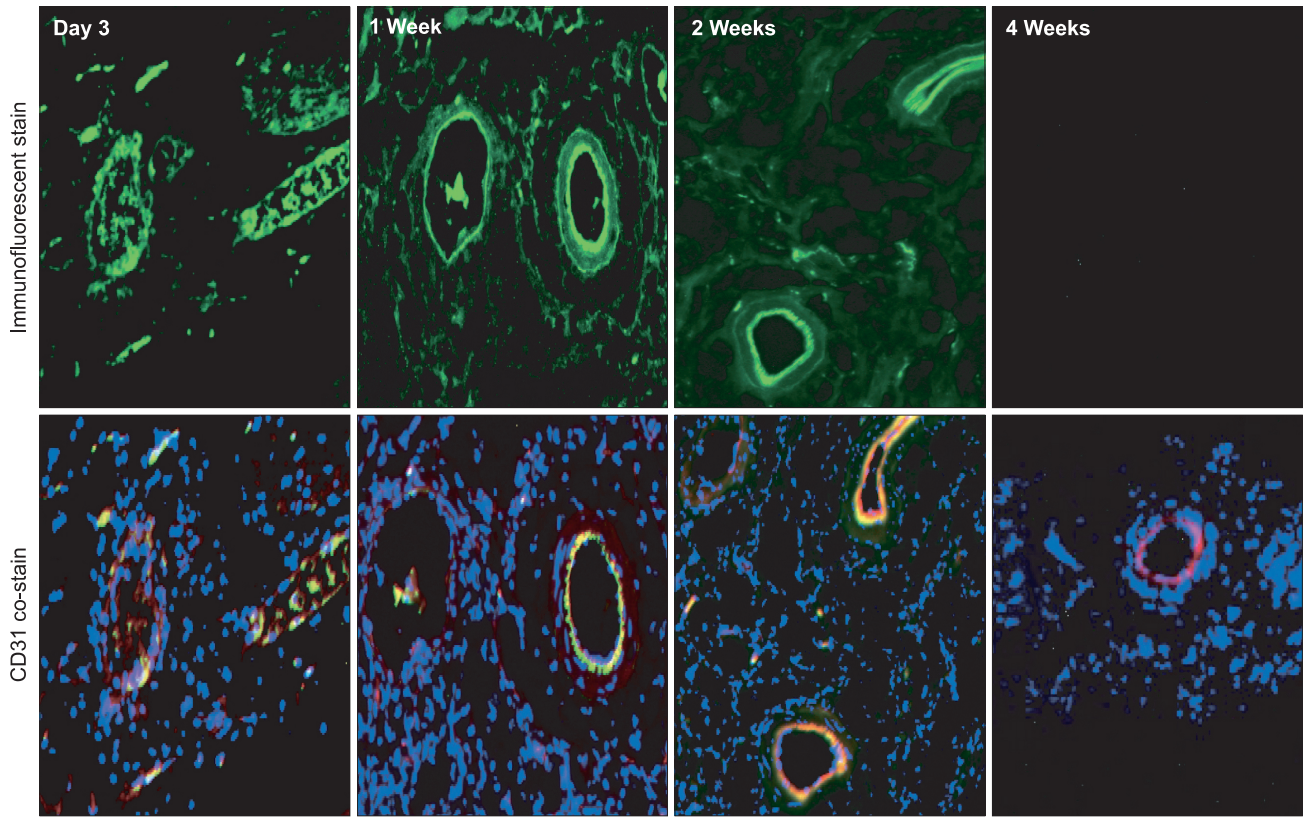

Fig. 2. Implantation of differentiated muscle-derived stem cells tagged with green fluorescence protein (MDSC-GFP) in blood vessels shown by immunoflurorescent staining of harvested hindlimb tissues at different time intervals (x100). Co-stain with endothelial marker CD31 shows characteristic yellow color derived from the merging of GFP (green) and CD31 (red). MDSC-GFP is no longer visible at 4 weeks.

both single stain and CD31 co-stain (only the red color of CD31 is visualized in the co-stain). Assessment of survival rate in this model showed that implantation was robust (0.61 ± 0.21 pixel/μm^2) at day 3 (1 day after injection), with a mild decrease in survival at 1 week (0.44 ± 0.10 pixel/μm^2), a substantial decrease at 2 weeks (0.16 ± 0.12 pixel/μm^2), and minimal GFP expressing cells were found at 4 weeks (0.01 ± 0.01 pixel/μm^2) (Fig. 3A). The quantified survival rate of GFP expressing cells normalized against day 3 was 72.0% at 1 week, 26.8% at 2 weeks and 2.4% at 4 weeks (Fig. 3B). In contrast, the control tissues showed no expression of GFP as expected.

3) Stem cell fate tracking in remote organs

Mobilization of injected MDSC-GFP to distant organs was assessed by harvesting the heart, liver and kidneys after intravascular implantation in the carotid arteries (Fig. 4). Immunofluorescent co-stain of GFP and CD31 in these organs showed that there was immediate expression of GFP positive cells in the heart and kidneys. This expression is no longer evident in the heart after 1 week, but GFP expressing cells remained in the kidney at 1 week and up to 4 weeks. Interestingly, the liver showed positive GFP cells at 1 week which remained up to 4 weeks.

DISCUSSION

In this study, MDSCs tagged with GFP were tracked for up to 4 weeks after injection into two different vascular disease models. The main findings demonstrate that MDSC-GFP were able to successfully implant at the site of injection after intramuscular delivery, with higher affinity to reside in blood vessels. However, there was a time-dependent decrease in stem cell activity at the site of injection with most of the cells not being able to be tracked after 4 weeks. Stem cells also showed a tendency to implant in distant organs after intravascular delivery, especially to the kidneys and the liver, with some cells remaining as long as 4 weeks in these distant organs.

We have previously demonstrated that MDSCs differentiated in vitro to the endothelial lineage were able to increase angiogenesis in ischemic hindlimbs of mice as shown by increased perfusion under laser Doppler, increased capillary density and increased expression of genes involved in angiogenesis (Vegf, Hif-1α and Egr-1) [11].

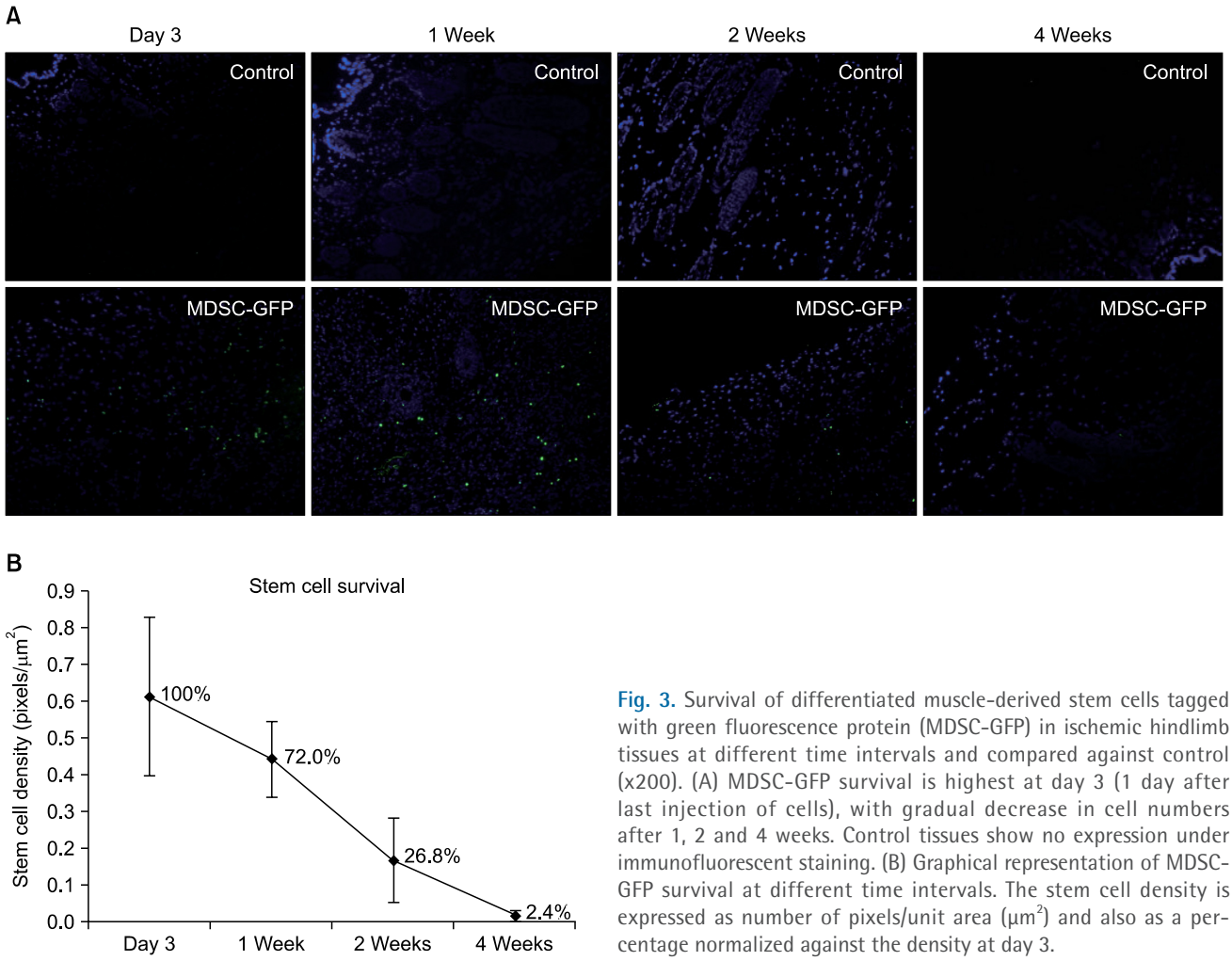

Fig. 3. Survival of differentiated muscle-derived stem cells tagged with green fluorescence protein (MDSC-GFP) in ischemic hindlimb tissues at different time intervals and compared against control (x200). (A) MDSC-GFP survival is highest at day 3 (1 day after last injection of cells), with gradual decrease in cell numbers after 1, 2 and 4 weeks. Control tissues show no expression under immunofluorescent staining. (B) Graphical representation of MDSC-GFP survival at different time intervals. The stem cell density is expressed as number of pixels/unit area (μm^2) and also as a percentage normalized against the density at day 3.

In this study, the high affinity of these cells to implant in blood vessels may partially explain for the increase in angiogenic activity. Since MDSC-GFP have positive expression of endothelial markers, the affinity for blood vessel endothelium may be very similar to the homing process observed in endothelial progenitor cells, mediated by important chemoattractants and adhesive molecules such as VEGF, stromal cell-derived factor (SDF)-1α, and P-/E-selectins [13]. The integration of stem cells into the vascular endothelium may also require transdifferentiation of a small fraction of these cells into smooth muscle cells, which may be mediated by growth factors (mainly platelet-derived growth factor-BB and Transforming growth factor-β1) and cell-cell or cell-matrix interactions [14-16]. These may be a few of the many complex factors that may constitute the niche involved in the migration of injected MDSC-GFP into blood vessels. Another very important factor is the hypoxic environment present in ischemic hindlimbs, which constitutes the environment of the target organ. Hypoxia is known to be a potent drive for angiogenesis, mainly by paracrine upregulation of angiogenic factors such as VEGF, HIF-1α and SDF-1 [17-19], and may explain for the affinity of MDSC-GFP to vascular endothelia.

Stem cells are known to self renew, by symmetric or asymmetric mechanisms, to generate either one or two daughter cells with similar developmental potential to the mother cell. In this ideal context, a stem cell should be able to maintain itself indefinitely. A previous study showed that MDSCs could be expanded beyond 200 population doublings in vitro, and their long-term self-renewal capacity was demonstrated in vivo by sequential injection, reharvesting and retransplantation of these cells [20]. These serially transplanted cells were able to regenerate skeletal muscle fibers in vivo, showing that their functions were also maintained. However in our study, injected stem cells decreased in numbers after 1 week and were not able to survive for more than 4 weeks at the site of injection. This decrease in number may partly be attributed to acute donor cell death, and it has been suggested that lack of matrix support, ischemia and inflammation all play important roles

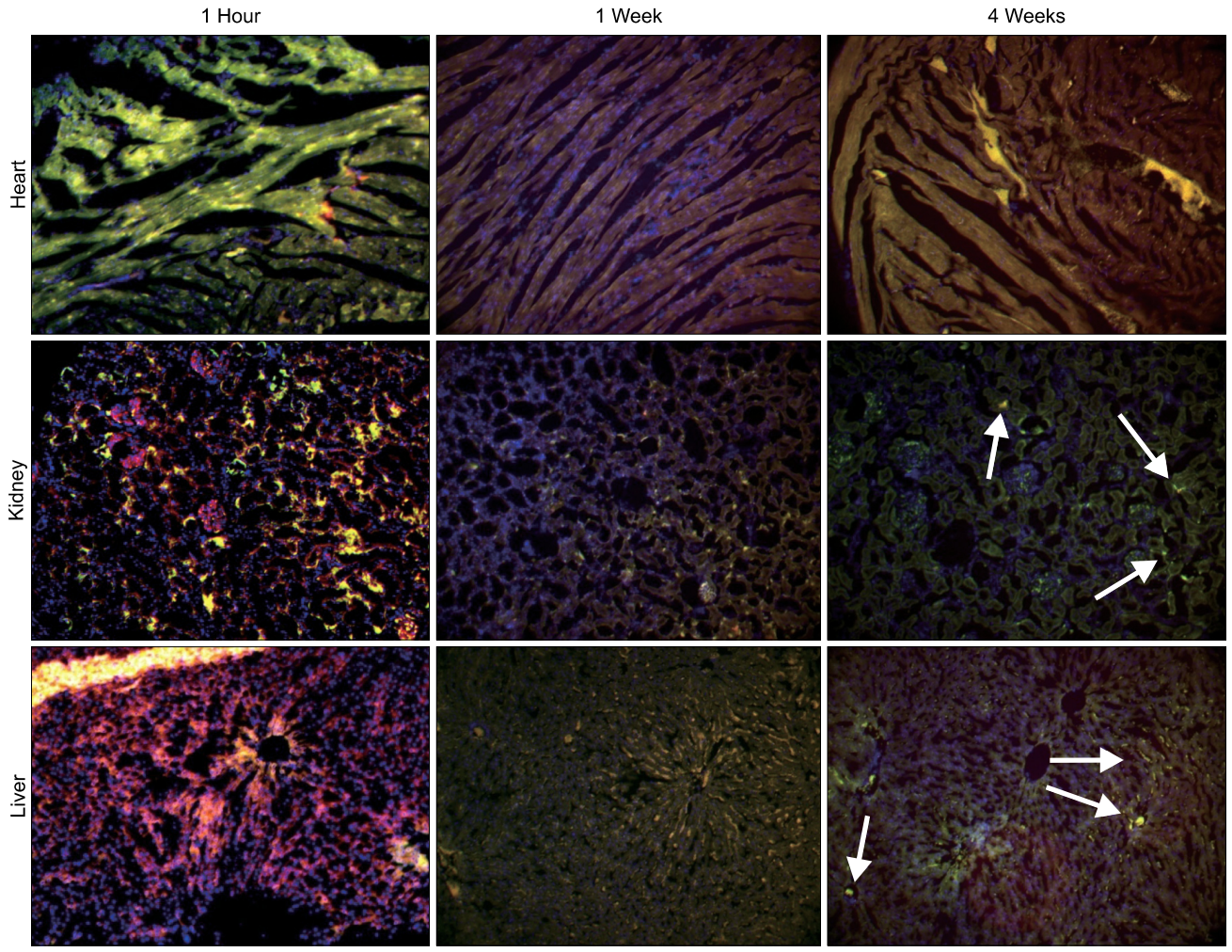

Fig. 4. Differentiated muscle-derived stem cells tagged with green fluorescence protein (MDSC-GFP) implantation in distant organs (x100). MDSC-GFP are found in the liver and kidney at 1 and 4 weeks under immunofluorescent co-stain with CD31, as shown by the positive expression (yellow color) of cells up to 4 weeks (arrows).

[21]. Injected cells tend to cluster and diffusion is the only way to obtain nutrient transport before angiogenesis can provide a vasculature. Therefore in ischemic hindlimbs, hypoxia may account for a certain proportion of early donor cell death before it induces angiogenesis. This ischemic site is also prone to inflammation, by recruitment of inflammatory cells and cytokines, which is able to initiate cell apoptosis [22]. Despite the decrease in stem cell survival, our previous results have shown highest capillary density and angiogenic expression under histologic analyses at 4 weeks, suggesting that the effects of stem cells are not directly correlated with cell numbers [11]. This may be explained by the fact that the most widely accepted mechanism of action of stem cells is through paracrine effects, although endocrine and autocrine pathways may also be involved. Therefore it is important to understand the interplay of autocrine, paracrine and endocrine mechanisms involved in cell fate regulation to improve our knowledge on niche conditions and enhance cell survival.

We have also observed stem cell implantation in distant organs, mainly the liver and kidneys, after intravascular stem cell incubation in the carotid injury model. Through intravascular delivery, there is a high proportion of cells circulating freely in the peripheral vascular system, since not all cells are able to successfully implant in the carotid endothelium during the 30 minute incubation period. Considering that the liver and kidneys are excretory organs with high vascularity, there is a higher probability that circulating cells may implant in these organs. However, the survival of these cells for more than 4 weeks suggests the possibility that certain niche conditions may exist in these organs that may allow them to implant rather than be excreted, or that endocrine effects may play a role in their distribution and mechanism of action. Therefore the method of delivery is

also important in determining the fate of stem cells, since the conditions these cells are immediately exposed to and the pattern of distribution may be different.

A limitation of this study is that different animal groups were used for comparison at different time intervals, which may be prone to bias. This may be overcome in future studies by use of bioluminescence imaging, since this method can detect signals in vivo and thus track stem cell survival at different intervals in the same animal, avoiding the need for multiple histological analyses. The importance of in vivo cell tracking methods for determining stem cell fate has been widely proposed, with newer imaging methods such as magnetic resonance imaging or radioisotope imaging and new labeling methods (reporter genes) being studied for larger-sized animals and humans [23,24]. Another limitation is that we did not track stem cell fate in distant organs after intramuscular injection into ischemic hindlimbs, limiting direct comparison between different modes of delivery (intramuscular vs. intravascular).

Overall, our results suggest that the problem of stem cell survival and sustained self-renewal need to be overcome in order to achieve prolonged effects. This requires a better understanding of cell- and tissue-specific niche conditions and the complex interactions that are involved, so that these conditions can be replicated both in vitro and in vivo. In this way, stem cells can be expanded efficiently, be driven to obtain desired effects, sustain their self-renewal capacity and ultimately avoid tumorigenesis. The use of tissue engineering technologies and epigenetic modulation techniques seem to be very promising for this purpose [6,25].

CONCLUSION

MDSCs differentiated in vitro towards the endothelial lineage and injected into different vascular disease models showed successful implantation at the site of injection with higher affinity to blood vessels, but there was a time-dependent decrease in survival. Methods to prolong cell survival for improved therapeutic effects are needed, which may require better knowledge of donor cell properties and interaction with host conditions (niche) to enhance stem cell self-renewal.

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