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CHAPTER VI

MISINTERPRETATION OF COCULTURE DIFFERENTIATION EXPERIMENTS BY UNINTENDED LABELING OF CARDIOMYOCYTES THROUGH SECONDARY TRANSDUCTION: DELUSIONS AND SOLUTIONS

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ABSTRACT

Cardiomyogenic differentiation of stem cells can be accomplished by coculture with cardiomyocytes (CMCs). To facilitate their identification, stem cells are often labeled through viral transduction with a fluorescent protein. A second marker to distinguish stem cell-derived CMCs from native CMCs is rarely used. This study aimed to investigate the occurrence of secondary transduction of unlabeled neonatal rat (nr) CMCs after coculture with human cells that had been transduced 0, 7 or 14 days earlier with a vesicular stomatitis virus (VSV) G protein-pseudotyped lentiviral vector (LV) encoding enhanced green fluorescent protein (GFP). To reduce secondary LV transfer, GFP-labeled cells were incubated with non-heat-inactivated human serum (NHI) or with VSV-neutralizing rabbit serum (α VSV). Heat-inactivated human serum (HI) and normal rabbit serum were used as controls. Immunostaining showed substantial *GFP* gene transfer to nrCMCs in cocultures started at the day of transduction indicated by the presence of GFP-positive/human lamin A/C-negative nrCMCs. The extent of secondary transduction was significantly reduced in cocultures initiated 7 days after *GFP* transduction, while it was completely abolished when human cells were added to nrCMCs 14 days post-transduction. Both NHI and α VSV significantly reduced the occurrence of secondary transduction compared to their controls. However, under all circumstances, GFP-labeled human cells had to be passaged for 14 days prior to coculture initiation to prevent any horizontal *GFP* gene transfer to the nrCMCs. This study emphasizes that differentiation experiments involving the use of viral vector-marked donor cells should be interpreted with caution and describes measures to reduce/prevent secondary transduction.

INTRODUCTION

Whether somatic stem cells (SSCs) can undergo cardiomyogenic differentiation without genetic intervention is a topic of much debate.^{1,2} At least part of the confusion may relate to application of different criteria and methods to identify SSC-derived cardiomyocytes (CMCs) and to the use of SSCs from different sources and differently aged donors.²⁻⁴ Intramyocardial transplantation and coculture with CMCs are commonly used to investigate cardiomyogenic differentiation potential of SSCs. However, these studies often do not include the use of species-, strain- or gender-specific markers to unambiguously demonstrate derivation of a CMC from a stem cell. Instead, stem cells are transduced with a viral vector encoding a fluorescent protein and the appearance of cells coexpressing the fluorescent protein and one or more CMC markers is taken as proof for their cardiomyogenic differentiation.⁵⁻¹¹ An often neglected pitfall of these studies is secondary transduction of CMCs by viral vector-marked stem cells. Pan *et al.* previously showed that hematopoietic target cell-associated vesicular stomatitis virus (VSV) G protein-pseudotyped lentiviral vector (LV) particles can transduce neighboring cells.¹² In this study, secondary transduction of neonatal rat (nr) CMCs by adult human cells that had previously been transduced with an enhanced green fluorescent protein (GFP)-encoding LV was studied. Also, options to abolish secondary transduction were investigated including incubation of LV-GFP-treated cells with VSV-specific antiserum or with normal human serum, which contains natural antibodies that can neutralize VSV in a complement-dependent manner.¹³

MATERIALS AND METHODS

Human tissues were obtained with donors' written informed consent and with approval of the Medical Ethics Committee of Leiden University Medical Center (LUMC). The study conformed to the principles of the Declaration of Helsinki. Animal experiments were approved by LUMC's Animal Experiments Committee and conformed to the Guide for Care and Use of Laboratory Animals (10236).¹⁴

Adult bone marrow (BM)- or adipose tissue (AT)-derived human mesenchymal stem cells (hMSCs) and human skin fibroblasts (hSFs) were transduced with GFP (multiplicity of infection 18 HeLa cell-transducing units/cell) using the VSV G protein-pseudotyped human immunodeficiency virus type 1 vector CMVPRES (hereinafter referred to as LV-GFP).^{15,16} This specific vector dose resulted in transduction efficiencies of nearly 100% without causing overt cytotoxicity. The aforementioned cell types and nrCMCs were isolated and cultured as previously described.¹⁷⁻¹⁹ nrCMC cultures typically contained $\pm 10\%$ cardiac fibroblasts and were mitomycin C-treated to prevent proliferation of the latter cell type.¹⁹ For a schematic overview

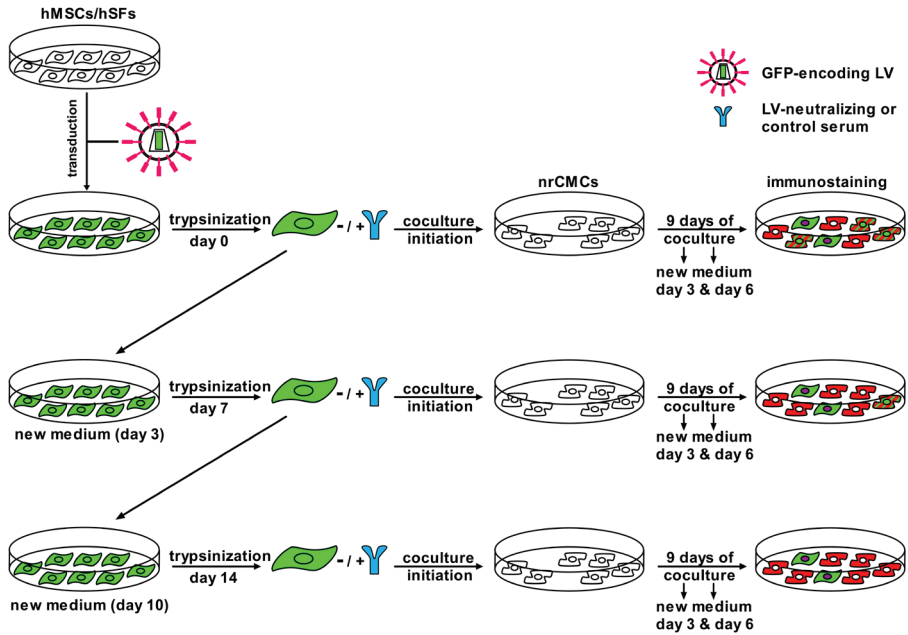


Figure 1. Schematic overview of the experimental setup. After incubation with LV-GFP for 4 hours, hMSCs or hSFs were washed three times with phosphate-buffered saline, detached using buffered 0.05% trypsin-0.02% EDTA solution and either immediately cocultured with nrCMCs (1:10 ratio) or subjected to an additional culture period of 7 or 14 days with 1 or 2 passages, respectively, prior to use in coculture. Medium was refreshed weekly. To reduce secondary transduction, GFP-labeled cells were incubated in NHI or α VSV for 1 h before start of coculture with nrCMCs. Controls were cells incubated with HI and normal rabbit serum, respectively. Neutralization experiments were conducted with freshly transduced human cells, but also with GFP-labeled cells that had been kept in monoculture for 7 or 14 days. After 9 days of coculture, cells were subjected to immunostaining. Abbreviations: LV-GFP, self-inactivating, vesicular stomatitis virus G-protein pseudotyped lentiviral vector coding for GFP; hMSCs, human mesenchymal stem cells; hSFs, human skin fibroblasts; nrCMCs, neonatal rat cardiomyocytes; GFP, enhanced green fluorescent protein; α VSV, vesicular stomatitis virus-neutralizing rabbit serum; NHI, non-heat-inactivated human serum; HI, heat-inactivated human serum.

of the experimental setup see Figure 1. After 9 days of coculture, cells were subjected to immunostaining as previously described.¹⁷ Primary antibodies specific for human lamin A/C (clone 636, VP-L550, Vector laboratories, Burlingame, CA) α -actinin (clone EA53, A7811) and connexin43 (C6219) (both from Sigma-Aldrich, Zwijndrecht, Netherlands) were used. These were visualized with Alexa Fluor-conjugated secondary antibodies, while nuclei were stained using Hoechst 33342 (all from Invitrogen, Breda, Netherlands). Image J software (Institutes of Health, Bethesda, MD) was used to determine the number of GFP-positive/human lamin A/C-negative nrCMCs in randomly chosen regions (³3,000 cells analyzed/condition). Human-specific quantitative RT-PCR analyses were used to detect possible cardiomyogenic differentiation of the human cells after 9 days of coculture with nrCMCs.¹⁷

Experimental results were expressed as mean $\pm$ standard deviation for a given number (n) of observations. Data was analyzed by Student's t-test for direct comparisons. Analysis of variance followed by appropriate post-hoc analysis was performed for multiple comparisons. Statistical analysis was performed using SPSS 16.0 for Windows (SPSS, Chicago, IL). Differences were considered statistically significant at $p < .05$.

RESULTS

Cocultures initiated with freshly transduced adult BM hMSCs, AT hMSCs or hSFs contained $51.2 \pm 2.1\%$, $40.6 \pm 3.8\%$ and $8.9 \pm 0.6\%$ of GFP-labeled nrCMCs, respectively (Figure 2A&D). GFP gene transfer to nrCMCs was strongly reduced in cocultures started with human cells 7 days post-transduction (BM hMSCs: $11.1 \pm 0.55\%$, AT hMSCs: $2.60 \pm 0.2\%$, hSFs: $2.10 \pm 0.1\%$; $p < .01$) (Figure 2B&D) and completely abolished using human cells that had been transduced 14 days earlier (Figure 2C&D). In none of the cocultures, expression of human *ACTN2*, *TNNT2*, *NPPA*, *MYL2*, *MYL7*, *GATA4* or *NKX2-5* was observed by quantitative RT-PCR confirming previous findings that hSFs and adult hMSCs do not undergo cardiomyogenesis when coincubated with nrCMCs (Figure 2E).¹⁷

Secondary transduction was inhibited by treatment of GFP-labeled adult BM hMSCs with non-heat-inactivated human serum (NHI) or VSV-neutralizing rabbit serum (α VSV) prior to coculture initiation. Compared to controls (heat-inactivated human serum and normal rabbit serum, respectively), incubation of the hMSCs with NHI or α VSV significantly reduced GFP gene transfer to nrCMCs in cocultures started at the day of transduction ($p < .01$) (Figures 3&4, A1, B1, C) or 7 days later ($p < .01$) (Figures 3&4, A2, B2, C), while hMSCs that had been transduced 14 days before coculture initiation did not give rise to any secondary transduction of nrCMCs (Figures 3&4, A3, B3, C).

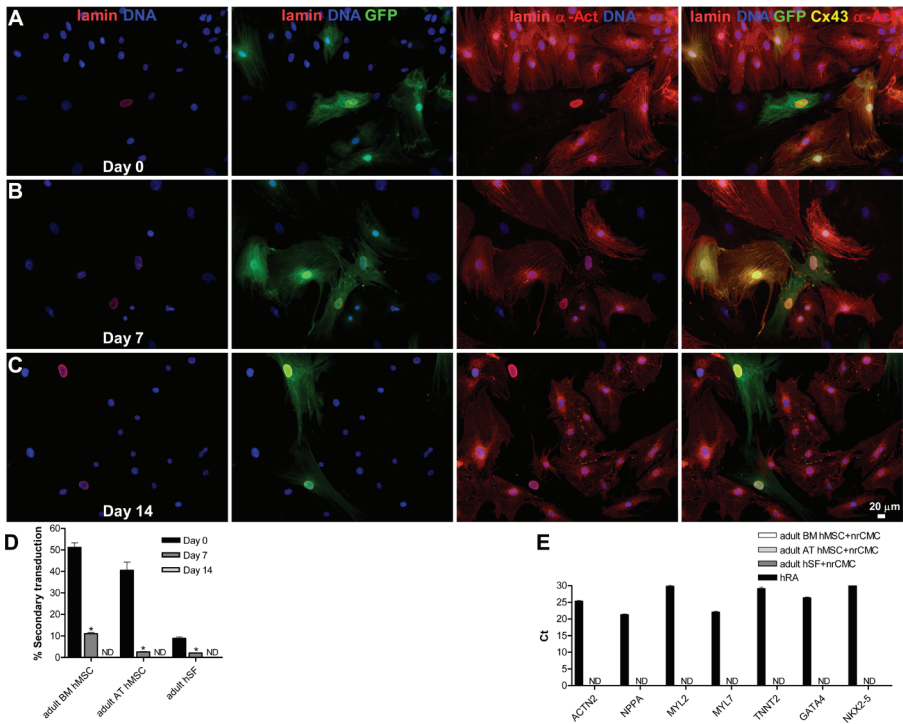


Figure 2. Secondary transduction of nrCMCs after coculture for 9 days with GFP-labeled human cells. (A): Typical fluorescence images of GFP- and human lamin A/C (lamin)-double-positive adult BM hMSCs and of cardiac α -actinin (α -Act)-positive nrCMCs in cocultures initiated at the day of transduction of the human cells with LV-GFP. A considerable number of GFP-positive/human lamin A/C-negative nrCMCs is present after 9 days of incubation indicating secondary transduction of nrCMCs. Cocultures were also stained for connexin43 (Cx43). Nuclei were stained with the DNA-binding fluorochrome Hoechst 33342. (B): In cocultures that were started 7 days after human cell transduction, adult BM hMSCs showed significant less horizontal GFP gene transfer to nrCMCs than those initiated with freshly transduced adult BM hMSCs. (C): Secondary transductions were not observed in mixed cultures of nrCMCs and LV-GFP-transduced adult BM hMSCs that had been passaged twice during a 14-day time period before coculture initiation. (D): Quantitative analysis of secondary transduction of nrCMCs in coculture with LV-GFP-transduced adult BM hMSCs, AT hMSCs or hSFs. The graph is based on a minimum of 3,000 cells analyzed per experimental group and time point. *, $p < .01$. (E): Human-specific qRT-PCR showed that none of the three human cell types expressed the cardiomyocyte marker genes *ACTN2*, *TNNI2*, *NPPA*, *MYL2*, *MYL7*, *GATA4* or *NKX2-5* after 9 days of coculture with nrCMCs. Human right atrium (hRA) samples were used as a positive control. Abbreviations: nrCMCs, neonatal rat cardiomyocytes; GFP, enhanced green fluorescent protein; BM, bone marrow; hMSCs, human mesenchymal stem cells; LV-GFP, self-inactivating, vesicular stomatitis virus G-protein pseudotyped lentiviral vector coding for GFP; AT, adipose tissue; hSFs, human skin fibroblasts; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; ND, not detected.

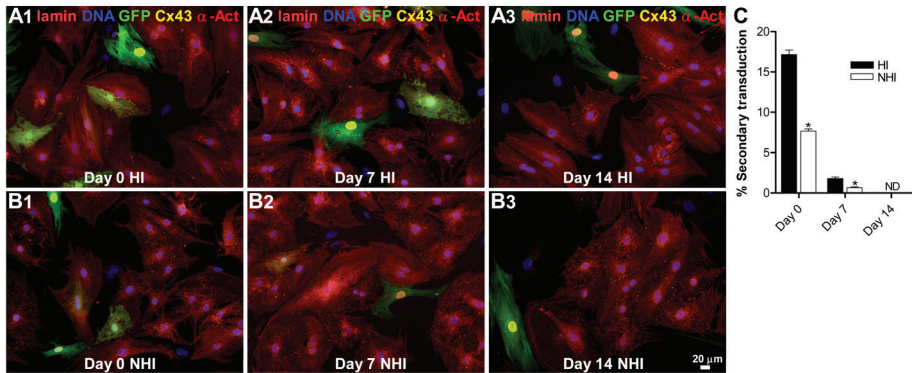


Figure 3. Secondary transduction of nrCMCs is reduced by incubating LV-GFP-transduced adult BM hMSCs with NHI prior to cocultivation with nrCMCs. (A1, B1): Typical fluorescence images of 9-day-old cocultures of GFP- and human lamin A/C (lamin)-double-positive cells with cardiac α -actinin (α -Act)-positive nrCMCs initiated at the day of transduction of the human cells with LV-GFP and following their treatment with NHI or HI. Incubation with NHI before coculture initiation leads to a significant reduction of horizontal *GFP* gene transfer to nrCMCs compared to incubation with HI as shown by a lower percentage of GFP-positive/human lamin A/C-negative nrCMCs (A2, B2): Treatment prior to coculture initiation of adult BM hMSCs that had been transduced 7 days earlier with LV-GFP with NHI resulted in less secondary transduction of nrCMCs than incubation with HI. Also, in cocultures started 7 days after exposure of adult BM hMSCs to LV-GFP the occurrence of secondary transduction was significantly reduced compared to those containing freshly transduced adult BM hMSCs. (A3, B3): Horizontal *GFP* gene transfer to nrCMCs was completely abolished in cocultures initiated 14 days after human cell transduction for both the NHI- and HI-treated adult BM hMSCs. Nuclei were stained with the DNA-binding fluorochrome Hoechst 33342. (C): Quantitative analysis of horizontal *GFP* gene transfer to nrCMCs in coculture with LV-GFP-labeled adult hMSCs that had been treated with NHI or HI before coculture initiation. The graph is based on a minimum of 3,000 cells analyzed per experimental group and time point. *, $p < .01$. Abbreviations: nrCMCs, neonatal rat cardiomyocytes; LV-GFP, self-inactivating, vesicular stomatitis virus G-protein pseudotyped lentivirus vector coding for enhanced green fluorescent protein; BM, bone marrow; hMSCs, human mesenchymal stem cells; GFP, enhanced green fluorescent protein; NHI, non-heat-inactivated human serum; HI, heat-inactivated human serum.

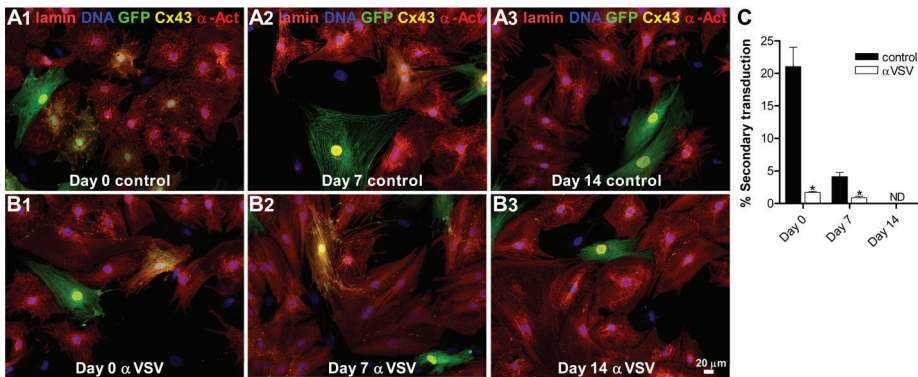


Figure 4. Secondary transduction of nrCMCs is reduced by incubating LV-GFP-transduced adult BM hMSCs with α VSV prior to coculture with nrCMCs. (A1, B1): Representative micrographs of 9-day-old cocultures of GFP- and human lamin A/C (lamin)-double-positive cells with cardiac α -actinin (α -Act)-positive nrCMCs initiated at the day of transduction of the human cells with LV-GFP and following their treatment with α VSV or normal rabbit serum (control). Incubation with α VSV before coculture initiation leads to a significant reduction of secondary transduction of nrCMCs compared to incubation with normal rabbit serum as shown by a lower percentage of GFP-positive/human lamin A/C-negative nrCMCs (A2, B2): Treatment prior to coculture initiation of adult BM hMSCs that had been transduced 7 days earlier with LV-GFP with α VSV resulted in less secondary transductions of nrCMCs than incubation with control serum. Also, in cocultures started 7 days after exposure of adult BM hMSCs to LV-GFP the occurrence of secondary transduction was significantly reduced compared to those containing freshly transduced adult BM hMSCs. (A3, B3): Horizontal GFP gene transfer to nrCMCs was completely abolished in cocultures initiated 14 days after human cell transduction for both the α VSV- and rabbit serum-treated adult BM hMSCs. Nuclei were stained with the DNA-binding fluorochrome Hoechst 33342. (C): Quantitative analysis of secondary transduction of nrCMCs in coculture with GFP-labeled adult BM hMSCs that had been treated with α VSV before coculture initiation. The graph is based on a minimum of 3,000 cells analyzed per experimental group and time point. *, $p < .01$. Abbreviations: nrCMCs, neonatal rat cardiomyocytes; LV-GFP, self-inactivating, vesicular stomatitis virus G-protein pseudotyped lentivirus vector coding for enhanced green fluorescent protein; BM, bone marrow; hMSCs, human mesenchymal stem cells; α VSV, vesicular stomatitis virus-neutralizing rabbit serum; GFP, enhanced green fluorescent protein.

DISCUSSION AND CONCLUSIONS

The key findings of this study are 1) hMSCs, and to a lesser extent, hSFs labeled with GFP by lentiviral gene transfer serve as a reservoir of functional LV particles causing secondary transductions of cocultured nrCMCs. 2) Passaging of the LV-GFP-transduced cells and frequent refreshment of culture medium prior to coculture initiation reduce and ultimately abolish GFP gene transfer to nrCMCs over time. 3) Incubation of the LV-GFP-transduced hMSCs with NHI or α VSV before coculture with nrCMCs greatly decreases GFP gene transfer to nrCMCs but does not shorten the time needed to completely eliminate secondary transduction. The strong inhibitory effect of NHI and α VSV on the frequency of GFP-labeled nrCMCs indicates that pseudo-transduction or vesicle uptake are not major contributors to the occurrence of GFP-positive nrCMCs in our coculture system.²⁰ Furthermore, the absence in the cocultures of human lamin A/C-positive cells expressing CMC markers excludes heterocellular fusion as cause for the appearance of GFP-labeled nrCMCs. Our results are in line with those of Pan *et al.*, who showed secondary transduction resulting from release of VSV G protein-pseudotyped LV particles by murine whole BM cells but did not investigate the effects of repeated passaging or treatment with α VSV or NHI of LV-transduced cells on horizontal gene transfer.¹²

CARDIOMYOGENIC DIFFERENTIATION VERSUS SECONDARY TRANSDUCTION

The ability of SSCs to undergo cardiomyogenesis remains a controversial topic. Coincubation with CMCs and intramyocardial transplantation are frequently used to investigate cardiomyogenic differentiation of SSCs.⁵⁻¹¹ In these experiments, the SSCs are often labeled with GFP using viral vectors before coincubation with nrCMCs and their cardiomyogenic differentiation is inferred from the occurrence of GFP-positive cells expressing CMC markers. In our laboratory, an antibody detecting human lamin A/C has been used as a second identifier besides GFP positivity to distinguish human cells from cocultured nrCMCs.^{17,18} Also, quantitative RT-PCR with human-specific primer pairs can be used to assess cardiomyogenesis in human cells cocultured with nrCMCs.^{17,18} Using this approach, human cardiomyocyte-specific gene expression was not observed even not under conditions that gave rise to high percentages of GFP- and α -actinin-double positive cells. Our finding that coincubation of LV-GFP-treated human cells with untransduced nrCMCs leads to considerable secondary transduction of the latter cells highlights the importance of using an endogenous marker to assess stem cell differentiation in the presence of the differentiated cell type(s) to be generated. This is particularly relevant when the combination of viral vector and target cell does not allow elimination of residual gene transfer activity by repeated cell passaging/prolonged cell culture as is the case when using episomal viral vectors (e.g. adenoviral vectors) or post-mitotic target cells.

DISCLOSURE

None.

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