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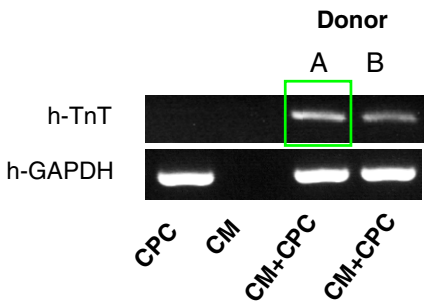
Genetic Proof-of-Concept for Cardiac Gene Expression in Human Circulating Blood-Derived Progenitor Cells

To the Editor: Transplantation of adult bone marrow– or blood-derived autologous progenitor cells is a promising option for treatment of patients with acute myocardial infarction or heart failure. Initial randomized, double-blind clinical trials demonstrated encouraging results suggesting that cell therapy improves heart function and patients' prognosis (1,2). However, the mechanisms underlying the beneficial effects of cell therapy are discussed controversially. Although most of the studies consistently reported an improvement of neovascularization by physical formation of capillaries and/or by paracrine effects (3), conflicting reports exist regarding the capacity of progenitor cells to differentiate into cardiomyocytes (4–7). Technical limitations leading to false-positive data have been accused of being at least in part responsible for the controversial studies. Studying a patient with a well-defined mutation in the cardiac troponin T (TnT) gene offered the unique opportunity to provide genetic proof for cardiac marker gene expression in patient-derived circulating cells after the induction of cardiac differentiation in vitro.

For this purpose, peripheral blood was collected from a 2-year-old patient with dilated cardiomyopathy caused by a heterozygous mutation of troponin T located on 1q32 exon 9 (G>A base exchange at position 8782) (8) (Online Fig. 1). The suspected mutation was verified in the explanted heart of the patient, who finally underwent heart transplantation after clinical decompensation. Circulating progenitor cells (CPC) were isolated by Ficoll density centrifugation of peripheral blood; mononuclear cells were incubated in endothelial basal medium with growth factors as described for endothelial progenitor cells (7,9) and cultivated for 14 days ex vivo. Cardiac differentiation was induced by a coculture system using neonatal rat cardiomyocytes as previously described (7,9). The CPC and cardiomyocytes were cocultured on gelatin-coated dishes for 6 days. Consistent with earlier reports, cocultured CPC expressed the cardiac marker protein alpha-sarcomeric actinin as detected by immunostaining (Online Fig. 2) and flow cytometry analysis ($4.1 \pm 0.5\%$ alpha-sarcomeric actinin$^+$/CM-DiI$^+$ cells; $n = 5$). Quantitative polymerase chain reaction (PCR) confirmed human TnT expression in cells after coculture with cardiomyocytes. The level of human TnT expression in the coculture was 4.6% compared with total human hearts (fluorescence intensity $4.58 \pm 0.68 - d(F1)/dT$ in control human heart and $0.21 \pm 0.07 - d(F1)/dT$ after coculture). To provide genetic evidence for cardiac marker gene expression, cells were harvested and reverse transcriptase (RT)-PCR was performed using human specific TnT primers. These primers were designed to span the region from exon 6 to exon 10 of human TnT to yield a 302-bp RT-PCR product (Online Fig. 1). Control samples confirmed the absence of the RT-PCR product when genomic deoxyribonucleic acid was used (data not shown). The 302-bp RT-PCR product was subcloned using pGEM-Teasy vector, and 7 clones were sequenced. As shown in Figure 1, the sequences of the cloned RT-PCR products were identical to human TnT except for the known G>A mutation at position 8782 in 3 of the 7 clones (Online Fig. 3). The incidence of the mutation (43%) is in line with the prediction of a heterozygous mutation.

To exclude cellular fusion between human CPC and neonatal rat cardiomyocytes as a mechanism for the induction of cardiac gene expression in the coculture assay, we used cardiomyocytes for coculture that were fixed with 1% paraformaldehyde. Fixed cardiomyocytes are unable to fuse but were previously shown to provide sufficient inductive signals to induce cardiac commitment of adult progenitor cells (7). After coculture of CPC with paraformaldehyde-fixed cardiomyocytes for 6 days, expression of TnT was again analyzed by RT-PCR. Human TnT expression was also detected in CPC after coculture with fixed cardiomyocytes as scaffold (Online Fig. 4), indicating that cardiac marker gene expression can occur independently from cellular fusion. The cardiac differentiation of CPC



Full Sequence of mutated clones

GAAGAAGAAGAGGAAGCAAAGGAGGCTGAAGAT
GGCCCAATGGAGGAGTCCAAACCAAAGCCCAGGT
CGTTCATGCCCAACTTGGTGCCTCCCAAGATCCC
CGATGGAGAGAGAGTGGACTTTGATGACATCCAC
CGGAAGCGCATGAAGAAGGACCTGAATGAGTTGC
AGGCGCTGATTGAGGCTCACTTTGAGAACAGGAA
GAAAGAGGAGGAGGAGCTCGTTTCTCTCAAAGAC
AGGATCGAGAGACGTCGGGCAGAGCGGGCCGAG
CAGCAGCGCATCCGGAATGAGCGGGAGAAGGA

Figure 1 **RT-PCR of Human TnT After Coculture of CPC With Rat Cardiomyocytes**

Reverse transcriptase-polymerase chain reaction (RT-PCR) with ribonucleic acid isolated from circulating progenitor cells (CPC) of 2 different donors after coculture with neonatal rat cardiomyocytes (CM) for 6 days. **(Top)** The RT-PCR product from the patient with the troponin T (TnT) mutation (donor A, **green box**) and the control patient (donor B) is shown. The CM or CPC without coculture were used as control samples to demonstrate the specificity of the human RT-PCR primers. The RT-PCR against human GAPDH (h-GAPDH) was used as loading control. The **green box** in the **bottom panel** indicates the sequence of the mutated TnT, with the primer sequence indicated in **blue** and the mutation site in **red**.

after coculture with fixed cardiac myocytes was further confirmed by detecting human messenger ribonucleic acid expression of the cardiac markers Nkx2.5, MLC-2v, and ANP (Online Fig. 4).

In summary, these experiments provide undisputable genetic evidence that circulating progenitor cells can be induced to express cardiac-specific genes. Although the number of cells acquiring a cardiac phenotype is rather low, the proof-of-concept that cardiac gene expression can be induced opens the possibility of enhancing cardiac differentiation capacity and thereby increasing the regenerative potential of circulating progenitor cells.

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APPENDIX

For supplementary figures, please see the online version of this article.