International Society for Cellular Therapy ISCT

Cytotherapy (2008) Vol. 10, No. 4, 376–389

informa healthcare

# Highly enriched cardiomyocytes from human embryonic stem cells

XQ Xu[1,2*], R Zweigerdt[1,2*], SY Soo[1,2], ZX Ngoh[1], SC Tham[1], ST Wang[1,2], R Graichen[1], B Davidson[1], A Colman[1,2] and W Sun[1,3]

[1]*ES Cell International Pte Ltd, Singapore,* [2]*Institute of Medical Biology, A*STAR, Singapore, and* [3]*Experimental Therapeutics Centre, A*STAR, Singapore*

**Background**

*Current efforts to direct differentiation of human embryonic stem cells (hESC) into a particular cell lineage usually lead to a heterogeneous cell population with only a fraction of the desired cell type present. We show the generation of an essentially pure population of human cardiomyocytes from hESC using lineage selection.*

**Methods**

*A construct comprising the murine α-myosin heavy chain (α-MHC) promoter driving the neomycin-resistance gene was introduced into hES3 cells to generate stable transgenic lines. Transgenic hESC lines were differentiated into cardiomyocytes and subjected to G418 selection. Both G418-selected and non-selected cardiomyocytes were characterized by immunocytochemistry and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. The teratoma-forming potential of differentiated cells was assessed by injection of about 2 million cells into the hind limb muscle of SCID mice.*

**Results**

*After cardiac differentiation and antibiotic selection in a suspension culture process, more than 99% of the transgenic cells showed immunoreactivity to α-MHC and α-actinin; this enrichment efficiency was observed for independent transgenic cell lines. Quantitative RT-PCR analysis revealed high levels of enrichment for cardiac-specific messages in the selected population. Importantly, injection of selected cells into six SCID mice resulted in no apparent teratoma formation, in contrast to differentiated but non-selected controls.*

**Discussion**

*Our results represent a significant step toward scalable production of pure human cardiomyocytes from stable, expandable hESC lines that will facilitate the development of cell therapies, safety pharmacology and drug discovery.*

**Keywords**

*arrhythmia, cardiomyocytes, cell therapy, differentiation, human embryonic stem cells (hESC), QT interval, safety pharmacology, selection, teratoma, transgenic lines.*

## *Introduction*

There is much current excitement surrounding the use of stem cells as a therapy for degenerative diseases such as congestive heart failure. Several stem or progenitor cell types are currently being evaluated in the clinic, including skeletal myoblasts, mesenchymal stromal cells and bone marrow-derived cells. However, no strong evidence exists that any of these cell types could form functional cardiomyocytes *in vitro* or *in vivo* [1]. Animal studies indicate that few if any of the nascent myotubes functionally couple to host cardiomyocytes following skeletal myoblast transplantation [2]. As for transplanted bone marrow cells, their differentiation into cardiomyocytes has been strongly questioned in recent publications [3–5]. In

*Correspondence to:* **William Sun**, Experimental Therapeutics Centre, 31 Biopolis Way, #03–01 Nanos, Singapore 138669; e-mail wsun@etc.a-star.edu.sg. **Xiu Qin Xu**, Institute of Medical Biology, 8A Biomedical Grove, # 06–06 Immunos, Singapore 138648; e-mail joya.xu@imb.a-star.edu.sg. **Robert Zweigerdt**, Institute of Medical Biology, 8A Biomedical Grove, # 06–06 Immunos, Singapore 138648; e-mail robert.zweigerdt@imb.a-star.edu.sg

*These authors contributed equally.



DOI: 10.1080/14653240802105307

contrast, embryonic stem cells (ESC) have been shown to be a promising cell source for generating large numbers of *bona fide* cardiomyocytes [6]. Recent studies have demonstrated that mouse and human (h) ESC-derived cardiomyocytes engraft long term into animal hearts, appear to couple functionally via gap junctions, and improve cardiac performance following transplantation into infarcted hearts [7–11].

Several groups have generated cardiomyocytes from hESC [12–15]. While most of the published protocols rely largely on spontaneous differentiation yielding only a small percentage of cardiomyocytes, improved strategies yielding more than 20% cardiomyocytes in serum-free media have been developed recently [16–18]. However, many of the envisioned applications would require essentially pure cardiomyocyte populations free of other cell lineages. In particular, residual, undifferentiated hESC that bear the inherent risk of teratoma formation after transplantation pose a key hurdle for the translation of hESC-derived lineages to clinical applications [19]. Pure cardiomyocyte populations having a defined, reproducible phenotype are also a pre-requisite to establish reliable novel assays for drug discovery and safety pharmacology.

To generate an enriched cardiomyocyte population from hESC, we used a lineage selection strategy by introducing a transgene designated myosin heavy chain (MHC)-neoR/pGK-hygR into hESC to form stable transgenic lines. The use of the murine α-MHC promoter to enrich for pure cardiomyocytes has been demonstrated previously in mouse ESC [8,9]. Alternative promoters, such as the myosin light chain 2v (MLC2v), have also been used successfully in mouse ESC to generate ventricular cardiomyocytes [20]. More recently, an MLC2v promoter was employed to control expression of an EGFP transgene in differentiated hESC, thereby enabling enrichment of human cardiomyocytes [21]. In addition, an antibiotic-based strategy utilizing a human α-MHC promoter to select hESC-derived cardiomyocytes has been published [22]. However, both studies have apparent limitations, which include the non-scalability of the differentiation and/or the selection procedures, utilizing pools of batch-transfected hESC instead of characterized transgenic hESC lines, achievement of suboptimal purity of the enriched cardiomyocyte cultures (maximum of 93% cardiomyocytes) and missing proof that the applied lineage selection is effective in preventing teratoma formation in respective animal models. We have addressed these issues by establishing stable, expandable hESC lines that allow the formation of highly pure human cardiomyocyte cultures in suspension that can be adapted to mass culture production, a process that has been established in recent mouse ESC studies [6,23]. Importantly, our strategy engineers a high level of safety into hESC-derived cell lineages that we have tested in a sensitive SCID mouse model.

## *Methods*

### ESC culture and transfection

hES3 cells (listed as HES-3 on the NIH Stem Cell Registry; http://stemcells.nih.gov/research/registry/ Accessed 1 January 2007) were cultured on mitotically inactivated human fibroblasts CCD919 (ATCC, Manassas, VA, USA). The hESC medium comprised KO-DMEM supplemented with 20% KO-SR, 100 μm non-essential amino acids, 2 mm l-glutamine and 0.5% v/v penicillin/streptomycin (all from Invitrogen, Carlsbad, CA, USA). For routine passaging, hES3 cells were split by manually cutting the colonies and transferring them to a new dish of human feeders. Prior to electroporation, hES3 cells were split by trypsin digestion for 4–6 passages. About 30 million cells were harvested and mixed with 40 μg linearized plasmid DNA MHC-neoR/pGK-hygR [8,9] in 0.8 mL hESC media. Cells were electroporated with a Bio-Rad GenePulser XL set to 320 V and 200 μF, essentially as described elsewhere [24,25]. Transfected hESC were plated at a density of 0.4 million cells/cm$^2$ on hygromycin-resistant mouse embryonic fibroblasts (Specialty Media, Millipore, Billerica, MA, USA). One week after plating, 50 μg/mL hygromycin were added to the cells. Emerging colonies were manually picked and expanded. G-banded karyotyping was performed by the Cytogenetics Laboratory at the KK Women's and Childrens' Hospital, Singapore.

### Southern blot analysis

Genomic DNA was extracted using Qiagen DNeasy columns. About 15 μg DNA was digested with *Eco*RI and separated on a 1% agarose gel. DNA was transferred to Hybond N+ membrane (Amersham, Piscataway, NJ, USA) and blotted with a dioxigenin-labeled probe against the mouse α-MHC promoter sequence. The probe was visualized with an anti-DIG antibody (Ab) conjugated to alkaline phosphatase (Roche Applied Science, Singapore) and chemiluminescent substrate CDP*Star (New England Biolabs, Ipswich, MA, USA).