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ORIGINAL ARTICLE

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# Chemically defined medium supporting cardiomyocyte differentiation of human embryonic stem cells

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**Abstract** Many applications of human embryonic stem cells (hESCs) will require fully defined growth and differentiation conditions including media devoid of fetal calf serum. To identify factors that control lineage differentiation we have analyzed a serum-free (SF) medium conditioned by the cell line END2, which efficiently induces hESCs to form cardiomyocytes. Firstly, we noted that insulin, a commonly used medium supplement, acted as a potent inhibitor of cardiomyogenesis in multiple hESC lines and was rapidly cleared by medium conditioning. In the presence of insulin or IGF-1, which also suppressed cardiomyocyte differentiation, the PI3/Akt pathway was activated in undifferentiated hESC, suggesting that insulin/IGF-1 effects were mediated by this signaling cascade. Time course analysis and quantitative RT-PCR revealed impaired expression of endoderm and mesoderm markers in the presence of insulin, particularly if added during early stages of hESC differentiation. Relatively high levels of the neural ectoderm marker Sox1 were expressed under these conditions. Secondly, comparative gene expression showed that two key enzymes in the prostaglandin I2 (PGI2) synthesis pathway were highly up-regulated in END2 cells compared with a related, but non-cardiogenic, cell line. Biochemical analysis confirmed 6–10-fold higher PGI2 levels in END2 cell-conditioned medium (END2-CM) vs. controls. Optimized concentrations of PGI2 in a fully synthetic, insulin-free medium resulted in a cardiogenic activity equivalent to END2-CM. Addition of the p38 mitogen-activated protein kinase-inhibitor SB203580, which we have shown previously to enhance hESC cardiomyogenesis, to these insulin-free and serum-free conditions resulted in a cardiomyocyte content of >10% in differentiated cultures without any preselection. This study represents a significant step toward developing scalable production for cardiomyocytes from hESC using clinically compliant reagents compatible with Good Manufacturing Practice.

**Key words** human embryonic stem cells · cardiomyocytes · insulin · prostaglandin $I_2$ · SB203580

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## Introduction

The ability to generate functional cardiomyocytes from human embryonic stem cells (hESCs) opens possibilities for developing more predictive human cardiac drug screens, disease models, and novel heart cell replacement technologies. Efficient methods of differentiation that supply homogeneous populations of cardiomyocytes of

sufficient quality, reproducibly, and in large quantities are a prerequisite for any of these applications. Future cell therapies will also require defined production protocols that meet regulatory requirements. However, the directed differentiation of hESCs toward cardiomyocytes is still poorly defined compared with the control of other cell fates and many methods rely on either the use of compliant serum batches, empirical use of recombinant growth factors, or cell co-culture systems (Kehat et al., 2001; Xu et al., 2002, 2006; Passier et al., 2005). Cost-effective, clinically compliant methods for the generation of cardiomyocytes from hESC have not yet been developed. Here, we have addressed this challenge by identifying factors present in a conditioned medium generated from a cell line that shows cardiogenic activity and have validated the contribution of these factors in a completely serum-free (SF) suspension culture assay.

Previous studies demonstrated directed differentiation of cardiomyocytes from mouse embryonic stem (ES) and embryonal carcinoma (EC) cells by co-culture with the mouse visceral endoderm-like cell line END2 derived from EC cells (Mummery et al., 1991; van den Eijnden-van Raaij et al., 1991). END2 cell co-culture later proved effective with hESCs, even in lines like HES2 and HES3 that previously appeared unable to form mesodermal derivatives, including cardiomyocytes, during the differentiation of embryoid body (EB)-like structures (Mummery et al., 2003). The use of SF medium increased the cardiogenic induction even further (Passier et al., 2005) and allowed sufficient cardiomyocytes to be produced for microarray analysis of the differentiation process itself (Beqqali et al., 2006) and for transplantation studies in mice that had undergone myocardial infarction (van Laake et al., 2007). Importantly, we have recently shown that efficient hESC cardiac differentiation can be made co-culture independent by utilizing a SF, END2 cell-conditioned medium (Dai et al., 2007; Graichen et al., 2008). Initial upscaling of this suspension culture approach has allowed cardiomyocyte generation for the transplantation into rat hearts (Dai et al., 2007) and even cell supply for functional testing in pigs that had undergone myocardial infarction (unpublished data) by adapting differentiation strategies we have previously established (Graichen et al., 2008). However, the nature of the cardiogenic activity secreted by END2 cells has remained elusive. Previous studies examining the biochemical properties of an END2 cell-conditioned medium suggested that it was likely to be protein based (van den Eijnden-van Raaij et al., 1991) but factors considered mesoderm inducers such as Activin A, FGFs, PDGFs, and Wnts, have been unable to replace this cardiac induction activity either in mouse EC cells or hESC (van den Eijnden-van Raaij et al., 1991; Passier et al., 2005).

In light of the importance of developing defined conditions for cardiomyogenesis, we have here combined biochemical and genomic approaches and identified both inducing and inhibitory activities important in mediating the END2 cell cardiogenic effect. We first deconstructed SF, END2 cell-conditioned medium (END2-CM) using a multi-well suspension culture that assayed cardiac differentiation of hESC as the end-point. This enabled systematic testing of media composition, media treatments, and common medium supplements. Using this approach we have shown that the SF medium supplement insulin was highly inhibitory for hESC cardiomyogenesis, and that END2 cells, as well as other cell types, were able to clear insulin effectively from the medium ensuring its absence in critical early stages of differentiation. We next used microarray analysis to identify potential candidate molecules by comparing differential gene expression in END2 cells vs. MES1 cells (Mummery et al., 1986, 1991), another differentiated mouse EC derivative that lacked cardiogenic activity entirely. Direct analysis indicated that prostaglandin $I_2$ (PGI2) was secreted at significantly higher levels by END2 cells than MES1 cells. The cardiogenic potential of PGI2 was evaluated in a synthetic, insulin-free medium and compared with END2-CM. Using the same assay, we further examined the cardiogenic activity of another small molecule, the specific p38 MAP kinase inhibitor SB203580, which was recently found to efficiently enhanced cardiomyocyte formation in combination with END2-CM (Graichen et al., 2008). Our findings have enabled us to develop a fully defined, cost-effective and non-xenogenic culture medium, which will be suitable for the scalable production of hESC cardiomyocytes.

## Material and methods

### hESC culture

hESC lines HES2 and HES3-GFP (Costa et al., 2005) from ES Cell International, http://stemcells.nih.gov/research/registry/esci.asp) at passages 75–125 and NL-HES1 and 2 (van de Stolpe et al., 2005) at passages 25–45 with normal karyotype were cultured in hESC medium: KO-DMEM, 20% Knockout™ serum replacement (KO-SR), 1 × NAA, 2 mM L-glutamine, bFGF (10 ng/ml; all Invitrogen, Carlsbad, CA) on human fibroblasts (CCD-919Sk, ATCC, Manassas, VA). Fibroblasts were cultured in DMEM, 20% FBS (Invitrogen), 2 mM L-glutamine, and 1 × ITS (insulin-tranferrin-selenium, Invitrogen) to confluence, treated with mitomycin C (10 μg/ml, Sigma, St. Louis, MO), and plated at a density of $3 \times 10^6$ cells/cm$^2$ for hESC culture. hESC were subcultured every 7 days using collagenase IV (1 mg/ml, Gibco Laboratories, Grand Island, NY) and mechanical slicing.

### Conditioned medium preparation and biochemical analysis

END2 cells were cultured as described previously (Mummery et al., 2003) using DMEM/F12 medium (Invitrogen) supplemented with 7.5% FBS (Invitrogen). SF medium comprised of DMEM, 1% MEM non-essential amino acids, 2 mM L-glutamine, 1 × ITS, and 0.1 mM β-mercaptoethanol (all Invitrogen) was added to start the conditioning process. END2-CM was collected after 1–7 days,