Published in final edited form as:

J Clin Exp Cardiol. 2012 April 25; Suppl 9: .

Defining Conditions for Sustaining Epiblast Pluripotency Enables Direct Induction of Clinically-Suitable Human Myocardial Grafts from Biologics-Free Human Embryonic Stem Cells

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Abstract

To date, lacking of a clinically-suitable human cardiac cell source with adequate myocardium regenerative potential has been the major setback in regenerating the damaged human myocardium. Pluripotent Human Embryonic Stem Cells (hESCs) proffer unique revenue to generate a large supply of cardiac lineage-committed cells as human myocardial grafts for cell-based therapy. Due to the prevalence of heart disease worldwide and acute shortage of donor organs or human myocardial grafts, there is intense interest in developing hESC-based therapy for heart disease and failure. However, realizing the potential of hESCs has been hindered by the inefficiency and instability of generating cardiac cells from pluripotent cells through uncontrollable multi-lineage differentiation. In addition, the need for foreign biologics for derivation, maintenance, and differentiation of hESCs may make direct use of such cells and their derivatives in patients problematic. Understanding the requirements for sustaining pluripotency and self-renewal of hESCs will provide the foundation for *de novo* derivation and long-term maintenance of biologics-free hESCs under optimal yet well-defined culture conditions from which they can be efficiently directed towards clinically-relevant lineages for therapies. We previously reported the resolving of the elements of a defined culture system, serving as a platform for effectively directing pluripotent hESCs uniformly towards a cardiac lineage-specific fate by small molecule induction. In this study, we found that, under the defined culture conditions, primitive endoderm-like (PEL) cells constitutively emerged and acted through the activin-A-SMAD pathway in a paracrine fashion to sustain the epiblast pluripotency of hESCs. Such defined conditions enable the spontaneous unfolding of inherent early embryogenesis processes that, in turn, aid efficient clonal propagation and *de novo* derivation of stable biologics-free hESCs from blastocysts that can be directly differentiated into a large supply of clinically-

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Citation: Parsons JF, Smotrich DB, Gonzalez R, Snyder EY, Moore DA, et al. (2011) Defining Conditions for Sustaining Epiblast Pluripotency Enables Direct Induction of Clinically-Suitable Human Myocardial Grafts from Biologics-Free Human Embryonic Stem Cells. *J Clin Exp Cardiol* S9:001. doi:10.4172/2155-9880.S9-001

Disclosures

The authors declare competing interests. XHP is the founder of Xcelthera. XHP and EYS have intellectual properties related to hESCs.

suitable human myocardial grafts across the spectrum of developmental stages using small molecule induction for cardiovascular repair.

Keywords

Small molecule induction; Defined culture system; Clonal propagation; bFGF; Insulin; Activin A; Nicotinamide; Human embryonic stem cell; Epiblast; Primitive endoderm; Pluripotency; Cardiac induction; Cardiac lineage specification; Cardiomesoderm; Cardioblast; Cardiac precursor; Cardiomyocyte; Myocardial graft; Human; Clinical; Cardiovascular repair; Myocardium regeneration

Introduction

Cardiovascular Disease (CVD) is a major health problem and the leading cause of death in the Western World. To date, lacking of a clinically-suitable human cardiac cell source with adequate myocardium regenerative potential has been the major setback in regenerating the damaged human myocardium, either by endogenous cells or by cell-based transplantation or cardiac tissue engineering [1-3]. The heart is the first organ formed from the cells of the Inner Cell Mass (ICM) or epiblast of the blastocyst in early embryogenesis. In the adult heart, the mature contracting cardiac muscle cells, known as cardiomyocytes, are terminally differentiated and unable to regenerate. Damaged or diseased cardiomyocytes are removed largely by macrophages and replaced by scar tissue. Although cell populations expressing stem/progenitor cell markers have been identified in postnatal hearts, the minuscule quantities and growing evidences indicating that they are not genuine heart cells have caused skepticism if they can potentially be harnessed for cardiac repair [3]. There is no evidence that stem/precursor/progenitor cells derived from other sources, such as bone marrow or cord blood, are able to give rise to the contractile heart muscle cells following transplantation into the heart [2,3]. Therefore, the need to regenerate or repair the damaged myocardium has not been met in today's healthcare industry [1-3].

Pluripotent Human Embryonic Stem Cells (hESCs), derived from the ICM or epiblast of the human blastocyst, proffer unique revenue to generate a large supply of cardiac lineage-committed cells as adequate human myocardial grafts for cell-based therapy [4,5]. Due to the prevalence of CVD worldwide and acute shortage of donor organs or adequate human myocardial grafts, there is intense interest in developing hESC-based therapy for heart disease and failure. The hESCs and their derivatives are considerably less immunogenic than adult tissues [2,3]. It is also possible to bank large numbers of human leukocyte antigen isotyped hESC lines so as to improve the likelihood of a close match [2,3]. However, realizing the developmental and therapeutic potential of hESCs has been hindered by the inefficiency and instability of generating cardiac cells from pluripotent cells through uncontrollable multi-lineage differentiation [5-8]. In addition, the need for foreign biologics for derivation, maintenance, and differentiation of hESCs may make direct use of such cells and their derivatives in patients problematic. In hESC-differentiating multi-lineage aggregates (embryoid bodies [EBs]), only a very small fraction of cells (< 4 %) spontaneously differentiate into cardiomyocytes [6-8]. With optimization and immune-selection, highly enriched populations of immature cardiomyocytes can be generated from hESCs [6-9]. Previously, it was shown that those hESC-derived enriched cardiomyocytes could generate small grafts and function as the biological pacemaker in animal infarcted models [10]. In rodent models of acute myocardial infarction, engrafted hESC-derived cardiomyocytes could survive transplantation to infarcted hearts of experimental animals and mature up to 12 weeks, and partially remuscularize the injured heart and improve the contractile function [8,11,12]. Although such hESC-derived cardiomyocytes can attenuate

the progression of heart failure in animal models of acute myocardial infarction, the grafts generated by cell transplantation have been small and insufficient to restore heart function or to alter adverse remodeling of a chronic myocardial infarction model following transplantation [8,11-13]. Tissue engineering has emerged as an alternate strategy recently; however, engineered tissue generated from preparations of hESC-derived cardiomyocytes survives poorly after transplantation, most likely due to ischemia [8]. Thus, developing strategies to channel the wide differentiation potential of pluripotent hESCs exclusively and predictably to a cardiac phenotype is vital to harnessing the power of hESC biology for cardiovascular repair.

The hESC lines were initially derived and maintained in co-culture with growth-arrested mouse embryonic fibroblasts (MEFs) [4]. Using this mouse-support system may compromise the therapeutic potential of these hESCs because of the risk of transmitting xenopathogens, altering genetic background, or promoting the expression of immunogenic proteins [14]. Alternatively, undifferentiated hESCs have been cultivated on exogenous human feeder layers [15], though the risk of transmitting human pathogens or other potential contaminants or immunogenic elements remains. Although several feeder-free culture systems have been suggested for hESCs, the issue of entirely eliminating contamination from exogenous undefined sources while sustaining prolonged stable growth remains unsolved [16-20]. So far, no reported normal clinically-suitable hESC line has been generated *de novo* under defined biologics-free conditions from its inception [19,20]. Without an understanding of the essential developmental components for sustaining hESC pluripotency and self-renewal, such hESC lines are at risk for becoming unhealthy and unstable after prolonged culturing under artificially-formulated chemically-defined conditions [19,20]. Maintaining undifferentiated hESCs in a defined biologics-free culture system that allows faithful expansion and controllable lineage-specific differentiation of safely engraftable cells is one of the keys to their therapeutic utility. Achieving this goal requires a better understanding of the minimal essential components necessary and sufficient for sustaining the pluripotent state and well-being of undifferentiated hESCs.

To tackle the shortcomings in conventional approaches, previously, we have resolved the elements of a defined culture system, serving as a platform for effectively directing such hESCs uniformly towards clinically-relevant lineages [20-22]. To achieve uniform conversion of pluripotent hESCs to a specific lineage, we have employed the defined culture system capable of insuring hESC proliferation to screen a variety of small molecules to identify conditions necessary for directing hESCs exclusively towards a lineage-specific fate without an intervening multi-lineage differentiation stage. We found that pluripotent hESCs maintained under the defined culture conditions can be uniformly converted into a specific lineage by small molecule induction [20-22]. Retinoic acid (RA) was found to induce the specification of neuroectoderm direct from the pluripotent state of hESCs and trigger progression to neuronal progenitors and neurons efficiently [20,21]. Similarly, nicotinamide (NAM) was found to induce the specification of cardiomesoderm direct from the pluripotent state of hESCs and trigger progression to cardiac precursors and cardiomyocytes efficiently [20,22]. This technology breakthrough enables well-controlled generation of a large supply of human myocardial grafts across the spectrum of developmental stages direct from the pluripotent state of hESCs with small molecule induction [20,22].

To formulate defined conditions for *de novo* derivation and long-term stable growth of biologics-free hESCs that are suitable for clinical applications likely requires knowledge regarding the minimal essential components necessary and sufficient for sustaining the pluripotent state and the well-being of undifferentiated hESCs. Therefore, in this study, we sought to identify the essential requirement for sustaining pluripotency and self-renewal of undifferentiated clonal hESCs. We found that such defined conditions derived their efficacy

from enabling the spontaneous unfolding of inherent early embryogenesis processes *in vitro* that emulated the maintenance of the pluripotent epiblast developed from the ICM *in vivo*. In early embryogenesis, the epiblast is composed of more matured pluripotent cells serving as the most immediate precursors of the early somatic lineages [23-25]. Under the defined culture conditions, primitive endoderm-like (PEL) cells constitutively emerged from their clonally-related precursors and acted through the activinA-SMAD pathway in a paracrine fashion to sustain the more progressed state of epiblast pluripotency of hESCs. Such a system emulating human embryonic development then served as a platform for *de novo* derivation and long-term stable growth of biologics-free hESCs that can be directly differentiated into a large supply of clinically-suitable human myocardial grafts for cardiovascular repair using small molecule induction.

Materials and Methods

Culture of undifferentiated hESCs

The hESC lines WA01 and WA09 (WiCell Research Institute) and newly-derived biologics-free hESCs (Xcel-hESCs) were used in this study. The defined culture systems consist of DMEM/F-12 or KODMEM (knockout-DMEM) (80%), Knockout Serum Replacement (KO) (20%), L-alanyl-L-gln or L-gln (2 mM), MEM nonessential amino acids (MNAA, 1X), β -Mercaptoethanol (β -ME, 100 μ M) (all from Invitrogen), human purified laminin (Sigma) or laminin/collagen (growth factor reduced Matrigel, BD Bioscience) as the matrix protein, and bFGF (basic fibroblast growth factor, 20 ng/ml) (PeproTech Inc). The KO can be replaced with defined essential factors containing MEM essential amino acids (MEAA, 1X), human insulin (20 μ g/ml) (Sigma), and ascorbic acid (50 μ g/ml) (Sigma), in which activin A (50 ng/ml, Sigma), human albumin (10 mg/ml, Sigma), and human transferrin (8 μ g/ml, Sigma) were added in order to increase cell survival and maintain normal shape and healthy colonies.

Passage of hESCs by dissection or trypsin treatment

hESC colonies estimated as having >80% morphologically undifferentiated cells were marked with a pen on the underside of the plate, separated from the surrounding feeder/differentiated cells, sliced into pieces, and detached from the tissue culture plate with a sterile plastic pipette tip. The dissected hESC colony pieces were pooled together, transferred to a fresh feeder layer or a laminin/collagen- or laminin-coated plate, and allowed to attach overnight. For trypsin-mediated passaging, hESCs maintained in the defined conditions were treated with trypsin/EDTA-HBS (0.25%) (Invitrogen) at 37°C for several minutes until the colonies rounded up. Trypsin was neutralized by adding trypsin inhibitor (0.8 mg/ml) (Invitrogen), and the cells were dispersed into a single cell suspension. These single cells were then allowed to seed on purified human laminin or laminin/collagen and cultivated in the defined hESC media containing bFGF (20 ng/ml). Culture medium was replaced every other day. The hESCs were passaged every 6-7 days at a split ratio of between 1:8 to 1:4.

Plate coating

Tissue culture plates were incubated with a commercially-available combination of laminin and collagen known as Matrigel (Growth factor reduced, BD Bioscience) [diluted 1:30 in cold DMEM/F-12] or purified human laminin (40 μ g/ml) (Sigma) [diluted in cold DMEM/F-12] at 4°C overnight.

Isolation of hESC-derived PEL cells

hESC-derived PEL cells were isolated from the differentiated cells surrounding the periphery of undifferentiated hESC colonies grown in feeder-free defined culture. A two-step mechanical/enzymatic treatment method was employed. First, all of the morphologically distinct hESC colonies were mechanically dissected away from the cultures. Then the remaining cells were lifted by brief treatment with 0.05% trypsin and then transferred to new Matrigel/laminin-coated plates containing hESC medium. The PEL cells were further purified by repeating the isolation procedure multiple times until no morphologically hESC-like cells were observed. Oct4 staining was used to confirm that no positive cells remained. The PEL cells were expanded and cryopreserved. The PEL cells were plated in gelatin pre-coated 60 mm plates or 6-well plates at a density of $1.7 \times 10^4/\text{cm}^2$ and inactivated by irradiation at 50 Gy using a ^{137}Cs gamma-irradiator before being used as feeder cells. To prepare PEL-conditioned medium (CM), growth-arrested PEL cells were seeded at 56,000 cells/ cm^2 in a gelatin-coated plate in 10% FBS/DMEM media, and then switched to hESC media for 24 hr for collecting the PEL-CM.

Cellular immunofluorescence

hESCs were grown in a 12- or 24-well plate for 6 or 7 days after seeding. The cells were fixed with 4% paraformaldehyde and blocked in PBS containing 0.2% Triton X-100 and 2% BSA. The cells were incubated with the primary antibody in 0.1% Triton X-100 in PBS at 4°C overnight, and then with secondary antibody (Invitrogen/ Molecular Probe) in the same buffer at room temperature for 45 min. After staining with DAPI, cells were visualized under an immunofluorescence and deconvolution microscope, and quantified by the image analysis software (Olympus). Primary antibodies to Oct-4, SSEA-1, SSEA-4, Tra-1-60, Tra-1-81, alkaline phosphatase, cMyc, Sox-2, Nkx2.5, α -actinin, GATA-4, GATA-6, cardiac troponin I, cardiac troponin T, activin-A, POSTN, CSPG2, LIF, STC-2, TGF β 2, and β -actin were from Santa Cruz Biotechnology.

Southern blot analysis of the transgene of eGFP-hESC clones

The eGFP-positive undifferentiated hESC colonies generated from sorted single cells per well were cultivated under the defined conditions to mature size, genomic DNA was extracted, and the number and location of copies of the transgene were analyzed by Southern blot with a GFP probe and detected by Alkphos direct labeling and CDP-star detection system (Amersham Biosciences).

RT-PCR

Total RNA was extracted using the RNAasy kit (Qiagen) and treated with RNase-free DNase according to the manufacturer's instructions. Then 1 μg of the total RNA was reversed transcribed to cDNA with SuperScript II and Oligo (dT) 12-18 primers according to the manufacturer's instructions (Gibco).

Teratoma formation

Undifferentiated hESCs after at least 3 months of derivation and culturing in the defined conditions, including clonal populations, were dissociated into small clumps and injected intramuscularly into six-week-old SCID female mice. After 6-to-10 weeks or when the mice became symptomatic, tumors were processed for histological analysis.

Cardiac lineage-specific differentiation of hESCs using small molecule induction

Undifferentiated hESCs maintained under the defined culture conditions were treated with NAM (10 mM) 3 days after seeding for 4-5 days, and then allowed to form floating cellular clusters (cardioblasts) in a suspension culture in the HESC MEDIUM for 4-5 days. After

permitting attachment to a tissue culture substrate, the cardioblasts were further treated with NAM (10 mM) for one more week and cultured in a differentiation media consisting of DMEM/F-12 (90%), defined FBS (Hyclone) (10%), and L-alanyl-L-gln or L-gln (2 mM). Beating cardiomyocytes were observed in about one week after withdrawal of NAM, increased in numbers with time. For electrophysiological recording, the beating cardiomyocyte clusters were transferred to a Warner RC-27 chamber, perfused with the above culture medium, and impaled with microelectrodes with 10M resistance when filled with 3 mol/L KCl. The tip of the recording microelectrode was positioned just outside of the active contracting area and the still images of the recording pipette position were captured with a Hamamatsu chilled CCD camera. The unipolar, extracellular recordings of field potentials, similar to the body surface electrocardiogram commonly observed in clinical practice, were detected using an Axon Multiclamp 700A amplifier in current clamp mode and the pCLAMP 9 software (Axon Instruments).

***De novo* derivation of stable biologics-free hESC lines in the defined culture system**

Embryos that were in excess from IVF procedures and otherwise destined for destruction were donated under IRB-approved guidelines that mandated informed consent, protection of the donor's identity, and strict ethical research practices. Frozen embryos were thawed and allowed to develop to the blastocyst stage. The blastocysts were manually-dissected and placed in the defined culture conditions described above for expansion. The blastocysts attached to the dish and the ICM continued to expand in defined culture with mitotically-inactivated hESC-derived PEL cells or activin-A (50 ng/ml) were added to provide paracrine support. The ICM outgrowth was manually dissected into small clumps of cells, which were replated in the same defined culture conditions. The resulting hESCs were maintained under the defined conditions for >50 passages (>12 months). The hESC colonies were assessed for the undifferentiated state by their expression of standard markers. Pluripotency was confirmed by teratoma formation following transplanted into SCID mice and stable karyotypes were analyzed by SKY karyotyping on a regular basis.

Results and Discussion

Resolving the essential elements for the growth of undifferentiated hESCs permits constitutively emergence of the primitive endoderm-like cells

Previously, we have reported that bFGF, insulin, and ascorbic acid are essential components of a defined culture system capable of insuring stable prolonged growth of undifferentiated hESCs that requires matrix protein laminin [20-22]. We found that, at the optimal concentrations of 20 ng/ml bFGF, 20 µg/ml insulin, and 50 µg/ml ascorbic acid in a base medium that consisted of DMEM/F-12 or KO-DMEM, L-alanyl-L-gln or L-gln (2 mM), MEM essential amino acids solution (1X), MEM nonessential amino acids solution (1X), β-Mercaptoethanol (100 µM) with human albumin (10 mg/ml) and human transferrin (8 µg/ml), ~ 70% of hESC colonies maintained on matrix proteins containing laminin displayed a highly compact morphology and expressed markers consistent with the undifferentiated state, including alkaline phosphatase, Oct-4, SSEA-4, Tra-1-60, Tra-1-81, for prolonged periods [20]. Omitting either bFGF or insulin induced differentiation, as indicated by the loss of Oct-4 and SSEA-4 expression with the onset of SSEA-1 expression [20] (Figure 1A). To facilitate further analysis, reporter eGFP+ hESCs constitutively expressing GFP were used [26]. Under our formulated defined culture system, we observed that hESCs began constitutively to develop into two morphologically distinct populations: small compact undifferentiated Oct-4-positive CD44-negative cells inside the colonies; and flat, spindle-shaped, Oct-4-negative CD44-positive cells that had migrated beyond the perimeter of the colonies (Figure 1B and 1C). Marker expression analysis by comparative RT-PCR indicated that those Oct-4-negative CD44-positive cells that had migrated outside of the colonies

down-regulated epiblast-associated genes (e.g., Oct-4, Sox2, and Nanog) and upregulated genes associated with the early extra-embryonic endoderm lineage (e.g., GATA 4/6, Sox 7, DAB2, and CoupII/II), but not with the trophoblast lineage (e.g., CDX2, EOMES, and hCG- α) (Figure 1D), suggesting that these peripheral cells that had migrated out from the hESCs colonies resembled the primitive endoderm (PE) cells that surround the emerging epiblast in the developing ICM of the blastocyst *in vivo* [23-25]. Therefore, next, we determined whether such PE-like (PEL) cells would segregate to the appropriate extra-embryonic endodermal layer if co-cultured with undifferentiated hESCs. Reporter eGFP+ hESCs constitutively expressing GFP were used to facilitate the co-culturing analysis [26]. We found that whether one created the chimeric aggregates by mixing enhanced green fluorescence protein (eGFP)-positive PEL cells (green) with non-fluorescent hESCs (white) or non-fluorescent PEL cells (white) with eGFP-positive hESCs (green) in suspension culture, the PEL cells established residence at the periphery of the chimeric and expressed endoderm-specific markers, such as AFP (Figure 2A). Finally, an important indication of PE identity is the ability of such cells to further differentiate into cells characteristics of parietal and visceral endoderm. We found that, when such PEL cells were subjected to well-established differentiating stimuli, such as BMP2 and ddcAMP [27], they further differentiated into visceral (e.g., AFP+, HNF α 4+) and parietal (e.g., Sox17+, TM+, HNF α 4+) endoderm-like cells (Figure 2B). The PE is one of the first cell lineages to emerge in the blastocyst *in vivo* and helps maintain the pluripotency of the epiblast from the developing ICM [23-25]; hence, the constitutive emergence of such a lineage from hESCs maintained under these defined culture conditions might provide the optimal milieu for maintaining the well-being and self-renewing undifferentiated state of the hESCs.

Defining the intrinsic paracrine support for the self-renewal of clonal hESCs enables sustaining epiblast pluripotency

Having affirmed the PE identity of these spontaneously-emerging cells from undifferentiated hESCs maintained under the defined culture, we next determined whether they provided paracrine support to pluripotent hESCs, mirroring the *in vivo* interaction between the PE and the human epiblast. Historically and intrinsic to the pluripotent state of hESCs, undifferentiated hESCs form the typical tightly packed colonies of small compact cells in culture and cannot be cloned efficiently from single cells [4,28]. Under standard culture conditions that employed feeders, MEF-conditioned media, or artificially-formulated chemically-defined conditions in which bFGF or activin has been used, passaging undifferentiated hESCs by trypsin dissociation reportedly kills nearly 100% of the undifferentiated cells [4,15-19,28]. In addition, the rare hESC colonies that survived or adapted to trypsin treatment had an unacceptably high rate of spontaneous differentiation (>50%) and developing abnormal karyotype (~10-20%) than the parent colonies [19,29,30]. However, of immediate advantage over previous standard culture conditions is that the defined culture conditions we formulated here supported the passage, seeding, and growth of single undifferentiated hESCs following trypsin dissociation (Figure 3A). Therefore, we further performed clonogenicity analyses based on colony-forming units [CFUs] under various culture conditions by quantifying the number of single hESCs seeded into a miniwell that can successfully expand into an Oct-4-positive colony. Reporter eGFP+ hESCs constitutively expressing GFP were used to facilitate the clonogenicity analysis [26]. Undifferentiated eGFP+ hESCs, maintained under these defined culture conditions, were dissociated into single cells by trypsin digestion and, following sorting, individually seeded into wells of multi-well dishes, one-cell-per-miniwell, to allow expansion into single undifferentiated hESC colony (Figure 3B). The efficiency of clonal propagation of undifferentiated hESCs under this defined culture system appeared to require PEL cells or conditioned medium (CM) from PEL cells (PEL-CM) as compared to those of hESCs maintained in previous feeder or feeder-free culture systems [4,15-19] (Figure 3B).

The observation that PEL-CM could substitute for the PEL cells suggested that they secreted soluble factors that provided essential paracrine support for the expansion of dissociated single pluripotent hESCs. Therefore, large scale proteomic profiling by MudPIT [31-33] was performed to detect proteins that were specifically expressed in hESC-derived PEL-CM but not in conditioned medium from human foreskin fibroblasts (HF-CM) as the control. The most notable putative protein factors we identified were POSTN, TGF β 2, LIF, STC2 and Activin-A, as confirmed by Western blot analysis (Figure 3C). Each of these factors was then tested individually to see if it could replace the paracrine support of PEL-CM in the clonogenicity assay. Only activin-A was found to support the clonal growth of undifferentiated hESCs in an efficiency comparable to that of PEL-CM in the defined culture system (Figure 3D). Furthermore, addition of specific inhibitors of the activin-A receptors (ActRIIA/B) or its signaling pathway (Smad2/3) into PEL-CM inhibited its ability to support the clonal growth of undifferentiated hESCs (Figure 3E). Taken together these results suggest that activin-A is the essential factor secreted by PEL cells that provides paracrine support for the self-renewal of single hESCs in this defined culture system. Activin-A, TGF β 2, and LIF have been previously shown to support ESC growth in high density cultures, but none of these alone has been sufficient to maintain clonal hESC self-renewal [18,34]. It is likely that bFGF and insulin are also stimulating IGF receptors, although IGF I and II alone were not sufficient for sustaining the self-renewal and pluripotency of hESCs [35]. Although neurotrophic factors have been reported to support efficient clonal expansion in MEF feeder-containing systems [36] in an efficiency comparable to that reported here, such factors did not appear to constitute one of the essential defined elements in this feeder-free, biologics-free system.

Our findings suggest that the defined culture system consisting of the essential elements, including bFGF (20 - 50 ng/ml), insulin (20 μ g/ml), ascorbic acid (50 μ g/ml), and laminin, when provided to undifferentiated hESCs, were sufficient and necessary to permit the spontaneous unfolding of an inherent embryogenesis-like process. PEL cells constitutively emerged which acted through the activinA-SMAD pathway in a paracrine fashion to sustain the more progressed state of epiblast pluripotency, emulating *in vitro* the process by which the human ICM-derived pluripotent epiblast is preserved *in vivo*.

***De novo* derivation of stable biologics-free hESCs using the defined culture system**

The need to provide exogenous undefined biological materials and xeno-feeder cells for the derivation, expansion, and differentiation of pluripotent hESCs has compromised their therapeutic potential and safety when administered to humans. So far, no reported normal clinically-suitable hESC line has been generated *de novo* under defined biologics-free conditions from its inception [19,20]. Having established an optimized set of defined culture conditions that emulate human embryonic development for the maintenance and clonal expansion of hESCs, we further sought to validate this defined culture system by demonstrating its ability to be used in the *de novo* derivation of long-term stable biologics-free hESCs which, from their inception, are maintained under these defined conditions, thus, suitable for clinical applications. Surplus embryos otherwise destined for destruction were donated under IRB-approved guidelines. Seven embryos were thawed and cultured initially, and two developed to the blastocyst stage (Figure 4A). The blastocysts hatched from the zona pellucida either spontaneously or with acid Tyrode's solution treatment [4, 19], and were placed in the defined culture conditions we formulated above for expansion, where hESC-derived PEL cells or activin A (50 ng/ml) were added to provide paracrine support (Figure 4B). The blastocysts then attached to the culture dish and their ICMs continued to expand in culture (Figure 4C). The representative biologics-free hESCs were derived in this defined culture system from the ICM of frozen blastocysts and subsequently maintained for > 50 passages (> 12 months). During which time, the hESC colonies retain their

undifferentiated state, as indicated by their expression of pluripotent markers, including alkaline phosphatase, Oct-4, SSEA-4, Tra-1-60, Tra-1-81 (Figure 4C); pluripotency, as indicated by their teratoma formation when transplanted into SCID mice (Figure 4D); and a normal stable diploid karyotype (Figure 4E). We have been employing this defined system to culture additional ICM-derived hESCs, including 5 from normal blastocysts and 2 from disease (trisomy)-bearing blastocysts. Our observations further support that this defined culture system indeed captures the essential conditions for maintaining the self-renewal of biologics-free pluripotent hESCs, including *de novo* derivation from their inception.

Direct induction of clinically-suitable human myocardial grafts from biologics-free hESCs

In order to achieve uniformly conversion of pluripotent hESCs to a lineage-specific fate, in previous reports, we have used the defined culture system to screen the differentiation inducing effect of a variety of small molecules and growth factors on the pluripotent state of hESCs [20-22]. We found that such defined conditions rendered small molecule NAM sufficient to induce the specification of cardiomesoderm direct from the pluripotent state of hESCs that further progressed human cardiac precursors and beating cardiomyocytes with high efficiency by promoting the expression of the earliest cardiac precursor marker Nkx2.5 [20,22]. Therefore, we further determined if this compound NAM was sufficient to induce a cascade of cardiac differentiation events direct from the pluripotent state of hESCs when applied to *de novo* derived biologics-free hESCs maintained under the defined culture system from their inception. Upon exposure of newly-derived biologics-free hESCs to NAM, all the cells within the colony underwent morphology changes to large differentiated phalloidin-positive cells that down-regulated Oct-4 and Sox-2 expression, and began expressing the cardiac specific transcription factor (Csx) Nkx2.5 and α -actinin (Figure 5A). These large differentiated cells continued to multiply and the colonies increased in size, consistent with their expression of cMyc that is implicated in cell proliferation [37], but not markers associated with other lineages, including Pdx1 and AFP [endoderm] and Map-2, GFAP, Pax6, and HNK1 [ectoderm] (Figure 5A). Increased intensity of Nkx2.5 was usually observed in areas of the colonies where cells began to pile up (Figure 5A). Nkx2.5 is an evolutionally conserved homeobox transcription factor indispensable for normal cardiac development and mutations of the gene are associated with human congenital heart diseases, the most common human birth defects [38]. Expression of Csx/Nkx2.5 is the earliest marker for heart precursor cells in all vertebrates so far examined and is essential for proper cardiac septation and formation/maturation of electrical conduction system [38-40]. In vertebrates, the onset and pattern of early Nkx2.5 expression roughly coincide with the timing and area of cardiac specification in early embryogenesis, and Nkx2.5 gene continues to be expressed through development in the heart [38-40]. Our observation suggested that NAM appeared to trigger the cardiac induction direct of the pluripotent state of biologics-free hESCs that might emulate the specification of cardiomesoderm in human embryonic cardiogenesis, consistent with our previous reports [20,22].

The cardiac-committed hESCs induced by NAM were then detached and allowed to form floating cellular clusters (cardioblasts) in a suspension culture to continue the cardiac differentiation process [20,22]. After permitting the cardioblasts to attach, beating cardiomyocytes began to appear with dramatically-increased efficiency (~ 50%) when compared to those from spontaneous differentiation without treatment as the control (Figure 5B). Cells within the beating cardiomyocyte clusters expressed markers characteristic of cardiomyocytes, including Nkx2.5, GATA-4, α -actinin, cardiac troponin I (cTnI), and cardiac troponin T (cTnT) (Figure 5B).

In conclusion, we have identified bFGF (at an optimal concentration of 20 ng/ml), insulin (20 μ g/ml), ascorbic acid (50 μ g/ml), laminin, and activin-A (50 ng/ml) as the minimal essential elements for sustaining pluripotency and self-renewal of clonal hESCs in a defined

culture system, serving as a platform for *de novo* derivation of therapeutically-suitable pluripotent hESCs that can be directly induced into large supplies of safely engraftable exclusively cardiac lineage-committed progenies across the spectrum of developmental stages with adequate myocardial regenerative potential for cardiovascular repair in the clinical setting. The efficacy of this defined culture system appears to derive from recapitulating the *in vivo* early embryogenic processes *in vitro*, including differentiation of the ICM into the epiblast and PE, the latter providing paracrine signaling to the former that enables not only clonal self-renewal, but also well-controlled efficient derivation of clinically-relevant somatic lineages direct from pluripotent hESCs by the simple provision of small molecules. Armed with the knowledge of these minimal essential requirements for sustaining epiblast pluripotency, we further demonstrated that this defined culture system can be used to establish biologics-free long-term karyotypically stable hESC lines *de novo* that can be directly converted into a large supply of clinically-useful human myocardial grafts for therapeutic applications. Further assessment of the therapeutic potential of such cells in preclinical models of heart diseases and clinical trials may offer critical insights not only into strategies for myocardium regeneration but also into fundamental aspects of human embryonic heart development.

Acknowledgments

XHP has been supported by National Institute of Health (NIH) grants from National Institute on Aging (NIHK01AG024496) and The Eunice Kennedy Shriver National Institute of Child Health and Human Development (NIHR21HD056530). No NIH resources were used in the generation of new hESCs or human embryo-based research.

Abbreviations

CFU	Colony-Forming Unit
CM	Conditioned Medium
CVD	Cardiovascular Disease
EB	Embryoid Body
eGFP	Enhanced Green Fluorescent Protein
ESC	Embryonic Stem Cell
GFP	Green Fluorescence Protein
hESC	Human Embryonic Stem Cell
HF	Human Fibroblast
ICM	Inner Cell Mass
IGF-I	Insulin-Like Growth Factor-I
IGF-II	Insulin-Like Growth Factor-II
MEF	Mouse Embryonic Fibroblast
NAM	Nicotinamide
PE	Primitive Endoderm
PEL	Primitive Endoderm Like
RA	Retinoic Acid

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Figure 1. Resolving the essential elements for the growth of undifferentiated hESCs permits constitutively emergence of the PEL cells

(A) Analysis of undifferentiated hESCs seeded on purified human laminin and cultivated in a serum-free defined medium. Undifferentiated hESCs, indicated by the expression of Oct-4 (**red**) and SSEA-4 (**red**), maintained in media containing both bFGF and insulin do not express SSEA-1 (**red**). Omitting either bFGF or insulin results in differentiation and SSEA-1 expression in cells. All cells are indicated by DAPI staining of their nuclei (**blue**).

(B) Representative eGFP+ hESC colonies showing that the defined culture system permits constitutively emergence of differentiated fibroblast-like cells (PEL) that migrate beyond the colonies (hESC) as compared to those maintained on MEFs as the control. **(C)**

Immunocytochemical analysis showing that the cell surface protein CD44 was expressed in PEL cells, but not in their clonally-related hESCs. **(D)** Comparative RT-PCR analysis showing that those PEL cells downregulated epiblast associated genes (Oct-4, Sox2 and Nanog) and upregulated genes associated with the early extraembryonic endoderm lineage (GATA 4/6, Sox 7, Coup1/II, and DAB2), but not with the trophoblast lineage (CDX2, EOMES, and hCG- α).

Figure 2. Confirming the PEL identity

(A) The PEL cells constitutively emerged from their clonally-related hESCs maintained under the defined culture conditions segregate to appropriate extra-embryonic layer location when co-culturing with undifferentiated hESCs. Whether one created the chimeric aggregates by mixing green-fluorescent PEL cells (eGFP+) with white-non-fluorescent hESCs or white-non-fluorescent PEL cells with green-fluorescent hESCs (eGFP+) in suspension culture, the PEL cells established residence at the periphery of the chimeric and expressed endoderm-specific markers, e.g., AFP (red). DAPI is blue. (B) RT-PCR analysis of early PE associated markers (GATA4/6 and SOX7) and late markers associated with differentiation into visceral (alpha-feto protein [AFP], transthyretin [TTR], HNFα4) and parietal (HNFα4, thrombomodulin [TM]) endoderm in PEL cells (PEL), undifferentiated hESCs (hESCs), and differentiated PEL cells treated with BMP2 and ddcAMP (Diff PEL). Immunocytochemical analysis of hESC-derived PEL cells differentiating into cells expressing visceral or parietal endoderm markers. Prior to differentiation, all the PEL cells were GATA6+ (red), but AFP-negative. Following treatment with BMP2 and ddcAMP, ~90% of cells become positive for HNFα4 (red) and TM (red) and ~10% of cells become positive for AFP (green). Note that the AFP+ cells (green) began to emerge from the GATA6+ (red) PEL cells such that the 2 markers now co-localized within the same cells.

Figure 3. The PEL cells provide paracrine support to their clonally-related hESCs by activinA-SMAD pathway

(A) Efficient trypsin dissociation and expansion of pluripotent hESCs under the defined culture conditions. hESCs were treated with trypsin and dissociated into single cell suspension. These single cells were then allowed to seed. Undifferentiated hESC colonies, as indicated by Oct-4 (red) expression, appeared 4-7 days after seeding. White arrows point to a single hESC (day 1 after seeding) and a single-cell-derived hESC colony (day 2-4 after seeding), which are shown at higher magnification in the insets. (B) Comparison of the clonal efficiencies (as assayed by the expansion of single hESCs in a miniwell into Oct4+ colonies [CFUs]) under various culture conditions, including previous conditions employing MEF or HF feeders, MEF- or HF-CM, or high dose of bFGF (100 ng/ml) and/or Noggin (500ng/ml) suggests that the defined conditions require the presence of irradiated clonally-related PEL cells or those cell conditioned media (PEL-CM) to support clonal expansion of single undifferentiated hESCs. The undifferentiated state of representative isolated eGFP+ hESC colonies, expanded from a sorted single cell deposited into a single miniwell in the presence of PEL cells or PEL-CM, is indicated by its Oct-4 expression. DAPI (blue) also visualizes the pre-existing irradiated wild type hESC-derived supporting PEL cells that are eGFP and Oct-4 negative. The clonal origin of isolated eGFP+ hESC colony is confirmed by the presence of a single viral insertion site for the transgene (arrow; Southern blot). Unengineered undifferentiated hESCs were used as a control. (C) Western blot analysis of HF- and PEL-CM confirm the expression of a group of proteins specifically detected in PEL-CM but not in HF-CM by proteomic profiling using MudPIT. (D) Clonogenicity analysis indicates that activin-A (50 ng/ml) supports the clonal growth of hESCs in an efficiency comparable to that of PEL-CM in the defined culture system. (E) Addition of specific inhibitors of the activin-A receptors (ActRIIA/B) or its signaling pathway (Smad2/3), including ligand neutralizing antibodies (anti-activin-A), soluble ActRIIA/B-FC receptors, or SB-431542, into PEL-CM inhibits its ability to support the clonal growth of undifferentiated hESCs.

Figure 4. The defined culture system is suitable for *de novo* derivation of stable biologics-free hESCs

(A) The embryos were thawed and allowed to develop through 8 cell (day 3) and morula (day 4) stages to blastocyst stage (day 5). (B) The blastocysts hatched from the zona pellucida either spontaneously or with acid Tyrode's solution treatment were placed in the defined culture to allow the ICM to expand while the trophoblast degenerated. (C) The ICM was further dissected and placed in the defined culture system for expansion. The cells were maintained in this defined system for >50 passages during which time hESCs in the colonies retained their undifferentiated compact morphology and expressed undifferentiated markers including alkaline phosphatase (red), Oct-4 (green), SSEA-4 (green), Tra-1-60 (red), Tra-1-81 (red). (D) The *de novo* derived biologics-free representative hESCs retained pluripotent as suggested by forming teratomas when transplanted into SCID mice, and (E) maintained a stably normal diploid karyotype for >50 passages.

Figure 5. Direct induction of clinically-suitable human myocardial grafts from biologics-free hESCs

(A) Upon exposure of undifferentiated biologics-free hESCs to NAM under the defined culture system, large differentiated phalloidin (**green**)-positive, cMyc (**red**)-positive, Oct-4 (**red**)-negative, and Sox-2 (**green**)-negative cells within the colony began to emerge. NAM-induced Oct-4-negative (**red**) cells began to express Csx/Nkx2.5 (**green**) and α -actinin (**red**), consistent with early cardiac differentiation. Progressively increased intensity of Nkx2.5 was usually observed in areas of the colony where cells began to pile up. These differentiated cells did not express markers for other lineages, including AFP (**red**), Pdx1 (**green**), Map-2 (**red**), GFAP (**green**), HNK1 (**red**), and Pax6 (**green**). All cells are indicated by DAPI staining of their nuclei (**blue**). (B) These biologics-free hESC-derived cardioblasts progress to beating cardiomyocytes with high efficiency. NAM-induced cardiac-committed biologics-free hESCs yielded beating cardiomyocytes with high efficiency, as assessed by the percentages of cellular clusters that displayed rhythmic contractions. Quantitative data are mean values from at least three separate experiments and at least 2 different cell lines. Germ-layer-induced hESCs without treatment were used as controls. The representative large beating cardiomyocyte clusters (phase) were immunopositive for Nkx2.5 (**green**), GATA-4 (**green**), α -actinin (**red**), cardiac troponin I (cTnI, **red**), cardiac troponin T (cTnT, **red**) (DAPI is **blue**).